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<b>(21) International Application Number:</b> PCT/US95/14598 <b>(22) International Filing Date:</b> 24 October 1995 (24.10.95) <b>(30) Priority Data:</b> 328,932 25 October 1994 (25.10.94) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US Not furnished (CON) Filed on Not furnished <b>(71) Applicant (for all designated States except US):</b> EMISPHERE TECHNOLOGIES, INC. [-US]; 15 Skyline Drive, Hawthorne, NY 10532 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MILSTEIN, Sam, J. [US/US]; 1 Knollwood Drive, Larchmont, NY 10538 (US). BARANTSEVITCH, Evgueni [RU/US]; Apartment 2B, 127 Cooper Drive, New Rochelle, NY 10801 (US). LEONE-BAY, Andrea [US/US]; 20 Woodland Way, Ridgefield, CT 06877 (US). WANG, Nai, Fang [CN/US]; Apartment 2C, 41-42 42nd Street, Long Island City, NY 11104 (US). SARUBBI, Donald, J. [US/US]; 18 Lawton	<b>(74) Agents:</b> ROBINSON, Joseph, R. et al.; Darby & Darby, P.C., 805 Third Avenue, New York, NY 10022 (US). <b>(81) Designated States:</b> AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> ACTIVE AGENT TRANSPORT SYSTEMS		
<b>(57) Abstract</b>  Methods for transporting a biologically active agent across a cellular membrane or a lipid bilayer. A first method includes the steps of: a) providing a biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native and denatured states; b) exposing the biologically active agent to a complexing perturbant to reversibly transform the biologically active agent to the intermediate state and to form a transportable supramolecular complex; and c) exposing the membrane or bilayer to the supramolecular complex, to transport the biologically active agent across the membrane or bilayer. The perturbant has a molecular weight between about 150 and about 600 daltons, and contains at least one hydrophilic moiety and at least one hydrophobic moiety. The supramolecular complex comprises the perturbant non-covalently bound or complexed with the biologically active agent. In the present invention, the biologically active agent does not form a microsphere after interacting with the perturbant. A method for preparing an orally administrable biologically active agent comprising steps (a) and (b) above is also provided as are oral delivery compositions. Additionally, mimetics and methods for preparing mimetics are contemplated.		

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## ACTIVE AGENT TRANSPORT SYSTEMS

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### FIELD OF THE INVENTION

The present invention relates to methods and compositions for transporting active agents, and particularly biologically active agents, across cell membranes or or lipid bilayers. These methods and compositions facilitate the  
15 delivery of an active agent to a target, such as the delivery of a pharmaceutical agent through an adverse environment to a particular location of the body.

### BACKGROUND OF THE INVENTION

Conventional means for delivering active agents to their intended  
20 targets, e.g. human organs, tumor cites, etc., are often severely limited by the presence of biological, chemical, and physical barriers. Typically, these barriers are imposed by the environment through which delivery must take place, the environment of the target for delivery, or the target itself.

Biologically active agents are particularly vulnerable to such  
25 barriers. Oral delivery to the circulatory system for many biologically active agents would be the route of choice for administration to animals if not for physical barriers such as the skin, lipid bi-layers, and various organ membranes that are relatively impermeable to certain biologically active agents, but which must be traversed before an agent delivered via the oral route can reach the  
30 circulatory system. Additionally, oral delivery is impeded by chemical barriers such as the varying pH in the gastrointestinal (GI) tract and the presence in the oral cavity and the GI tract of powerful digestive enzymes.

Calcitonin and insulin exemplify the problems confronted in the art in designing an effective oral drug delivery system. The medicinal properties of

calcitonin and insulin can be readily altered using any number of techniques, but their physicochemical properties and susceptibility to enzymatic digestion have precluded the design of a commercially viable delivery system. Others among the numerous agents which are not typically amenable to oral administration are  
5 biologically active proteins such as the cytokines (e.g. interferons, IL-2, etc); erythropoietin; polysaccharides, and in particular mucopolysaccharides including, but not limited to, heparin; heparinoids; antibiotics; and other organic substances. These agents are also rapidly rendered ineffective or are destroyed in the GI tract by acid hydrolysis, enzymes, or the like.

10 Biotechnology has allowed the creation of numerous other compounds, of which many are in clinical use around the world. Yet, the current mode of administration of these compounds remains almost exclusively via injection. While in many cases oral administration of these compounds would be preferable, these agents are labile to various enzymes and variations  
15 in pH in the GI tract and are generally unable to penetrate adequately the lipid bilayers of which cell membranes are typically composed. Consequently, the active agent cannot be delivered orally to the target at which the active agent renders its intended biological effect.

Typically, the initial focus of drug design is on the physiochemical  
20 properties of pharmaceutical compounds and particularly their therapeutic function. The secondary design focus is on the need to deliver the active agent to its biological target(s). This is particularly true for drugs and other biologically active agents that are designed for oral administration to humans and other animals. However, thousands of therapeutic compounds are discarded because  
25 no delivery systems are available to ensure that therapeutic titers of the compounds will reach the appropriate anatomical location or compartment(s) after administration and particularly oral administration. Furthermore, many existing therapeutic agents are underutilized for their approved indications because of constraints on their mode(s) of administration. Additionally, many  
30 therapeutic agents could be effective for additional clinical indications beyond those for which they are already employed if there existed a practical methodology to deliver them in appropriate quantities to the appropriate biological targets.

Although nature has achieved successful inter- and intra-cellular transport of active agents such as proteins, this success has not been translated to drug design. In nature, the transportable conformation of an active agent such as a protein is different than the conformation of the protein in its native state. In addition, natural transport systems often effect a return to the native state of the protein subsequent to transport. When proteins are synthesized by ribosomes, they are shuttled to the appropriate cellular organelle by a variety of mechanisms e.g. signal peptides and/or chaperonins. Gething, M-J., Sambrook, J., *Nature*, 355, 1992, 33-45. One of the many functions of either the signal peptides or the chaperonins is to prevent premature folding of the protein into the native state. The native state is usually described as the 3-dimensional state with the lowest free energy. By maintaining the protein in a partially unfolded state, the signal peptides or the chaperonins facilitate the protein's ability to cross various cellular membranes until the protein reaches the appropriate organelle. The chaperonin then separates from the protein or the signal peptide is cleaved from the protein, allowing the protein to fold to the native state. It is well known that the ability of the protein to transit cellular membranes is at least partly a consequence of being in a partially unfolded state.

Current concepts of protein folding suggest that there are a number of discrete conformations in the transition from the native state to the fully denatured state. Baker, D., Agard, D.A., *Biochemistry*, 33, 1994, 7505-7509. The framework model of protein folding suggests that in the initial early stages of folding the domains of the protein that are the secondary structure units will form followed by the final folding into the native state. Kim, P.S., Baldwin, R.L., *Annu. Rev. Biochem.*, 59, 1990, 631-660. In addition to these kinetic intermediates, equilibrium intermediates appear to be significant for a number of cellular functions. Bychkova, V.E., Berni, R., et al, *Biochemistry*, 31, 1992, 7566-7571, and Sinev, M.A., Razgulyaev, O.I., et al, *Eur. J. Biochem.*, 1989, 180, 61-66. Available data on chaperonins indicate that they function, in part, by keeping proteins in a conformation that is not the native state. In addition, it has been demonstrated that proteins in partially unfolded states are able to pass through membranes, whereas the native state, especially of large globular

proteins, penetrates membranes p orly, if at all. Haynie, D.T., Freire, E.,  
Proteins:Structure, *Function and Genetics*, 16, 1993, 115-140.

Similarly, some ligands such as insulin which are unable to underg  
conformational changes associated with the equilibrium intermediates described  
5 above, lose their functionality. Hua, Q. X., Ladbury, J.E., Weiss, M.A.,  
*Biochemistry*, 1993, 32, 1433-1442; Remington, S., Wiegand, G., Huber, R.,  
1982, 158, 111-152; Hua, Q. X., Shoelson, S.E., Kochoyan, M. Weiss, M.A.,  
*Nature*, 1991, 354, 238-241.

Studies with diphtheria toxin and cholera toxin indicate that after  
10 diphtheria toxin binds to its cellular receptor, it is endocytosed, and while in this  
endocytic vesicle, it is exposed to an acidic pH environment. The acidic pH  
induces a structural change in the toxin molecule which provides the driving  
force for membrane insertion and translocation to the cytosol. See, Ramsay, G.,  
Freire, E. *Biochemistry*, 1990, 29, 8677-8683 and Schon, A., Freire, E.,  
15 *Biochemistry*, 1989, 28, 5019 - 5024. Similarly, cholera toxin undergoes a  
conformational change subsequent to endocytosis which allows the molecule to  
penetrate the nuclear membrane. See also, Morin, P.E., Diggs, D., Freire, E.,  
*Biochemistry*, 1990, 29, 781-788.

Earlier designed delivery systems have used either an indirect or a  
20 direct approach to delivery. The indirect approach seeks to protect the drug  
from a hostile environment. Examples are enteric coatings, liposomes,  
microspheres, microcapsules. See, colloidal drug delivery systems, 1994, ed.  
Jorg Freuter, Marcel Dekker, Inc.; U.S. Patent No. 4,239,754; Patel et al.  
(1976), *FEBS Letters*, Vol. 62, pg. 60; and Hashimoto et al. (1979),  
25 *Endocrinology Japan*, Vol. 26, pg. 337. All of these approaches are indirect in  
that their design rationale is not directed to the drug, but rather is directed to  
protecting against the environment through which the drug must pass enroute  
to the target at which it will exert its biological activity, i.e. to prevent the  
hostile environment from contacting and destroying the drug.

30 The direct approach is based upon forming covalent linkages with  
the drug and a modifier, such as the creati n f a prodrug. Balant, L.P.,  
Doelker, E., Buri, P., *Eur. J. Drug Metab. And Pharmacokinetics*, 1990, 15(2),  
143-153. The linkage is usually designed to be broken under defined

circumstances, e.g. pH changes or exposure to specific enzymes. The covalent linkage of the drug to a modifier essentially creates a new molecule with new properties such as an altered log P value and/or as well as a new spatial configuration. The new molecule has different solubility properties and is less susceptible to enzymatic digestion. An example of this type of method is the covalent linkage of polyethylene glycol to proteins. Abuchowski, A., Van Es, T., Palczuk, N.C., Davis, F.F., *J. Biol. Chem.* 1977, 252, 3578.

Broad spectrum use of prior delivery systems has been precluded, however, because: (1) the systems require toxic amounts of adjuvants or inhibitors; (2) suitable low molecular weight cargos, i.e. active agents, are not available; (3) the systems exhibit poor stability and inadequate shelf life; (4) the systems are difficult to manufacture; (5) the systems fail to protect the active agent (cargo); (6) the systems adversely alter the active agent; or (7) the systems fail to allow or promote absorption of the active agent.

There is still a need in the art for simple, inexpensive delivery systems which are easily prepared and which can deliver a broad range of active agents to their intended targets, especially in the case of pharmaceutical agents that are to be administered via the oral route.

## **SUMMARY OF THE INVENTION**

The present invention discloses methods for transporting a biologically active agent across a cellular membrane or a lipid bilayer. A first method includes the steps of:

(a) providing a biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native and denatured states;

(b) exposing the biologically active agent to a complexing perturbant to reversibly transform the biologically active agent to the intermediate state and to form a transportable supramolecular complex; and

(c) exposing the membrane or bilayer to the supramolecular complex, to transport the biologically active agent across the membrane or bilayer.

The perturbant has a molecular weight between about 150 and about 600 daltons, and contains at least one hydrophilic moiety and at least one hydrophobic moiety. The supramolecular complex comprises the perturbant non-covalently bound or complexed with the biologically active agent. In the present invention, the biologically active agent does not form a microsphere after interacting with the perturbant.

Also contemplated is a method for preparing an orally administrable biologically active agent comprising steps (a) and (b) above.

In an alternate embodiment, an oral delivery composition is provided. The composition comprises a supramolecular complex including:

- (a) a biologically active agent in an intermediate conformational state which is reversible to the native state, non-covalently complexed with
- (b) a complexing perturbant having a molecular weight ranging from about 150 to about 600 and having at least one hydrophilic moiety and at least one hydrophobic moiety;

wherein the intermediate state is conformationally between the native conformation state and denatured conformation state of the biologically active agent and the composition is not a microsphere.

Further contemplated is a method for preparing a mimetic which is transportable across cellular membrane(s) or lipid-bilayer(s) and which is bioavailable to the host after crossing the membrane(s) or bilayer(s). A biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native state and the denatured state, is exposed to a complexing perturbant to reversibly transform the biologically active agent to the intermediate conformational state and to form a transportable supramolecular complex. The perturbant has a molecular weight between about 150 and about 600 daltons and at least one hydrophilic moiety and one hydrophobic moiety. The supramolecular complex comprises the perturbant non-covalently complexed with the biologically active agent, and the biologically active agent does not form a microsphere with the perturbant. A mimetic of the supramolecular complex is prepared.



Alternatively, a method for preparing an agent which is transportable across a cellular membrane or a lipid-bilayer, and which is bioavailable after crossing the membrane or bilayer, is provided. A biologically active agent which can exist in a native conformational state, a denatured conformational state, and an intermediate conformational state which is reversible to the native state and which is conformationally between the native and denatured states, is exposed to a perturbant to reversibly transform the biologically active agent to the intermediate state. The agent, a mimetic of the intermediate state, is prepared.

10

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is illustration of a native gradient gel of  $\alpha$ -interferon (IFN) and a modified amino acid complexing perturbant.

Figure 2 is an illustration of a native gradient gel of  $\alpha$ -interferon and a thermal condensate complexing perturbant.

Figure 3 is a graphic illustration of serum levels of  $\alpha$ -interferon after oral administration of  $\alpha$ -interferon with and without complexing perturbants.

Figure 4 is a graphic illustration of changes in serum calcium in rats orally administered salmon calcitonin with and without complexing perturbants.

Figure 5 is a graphic illustration of guanidine hydrochloride (GuHCl) induced denaturation of  $\alpha$ -interferon.

Figure 6 is a graphic illustration of the concentration effect of GuHCl on  $\alpha$ -interferon conformation.

Figure 7 is a graphic illustration of the pH denaturation of  $\alpha$ -interferon.

Figure 8 is a graphic illustration of the pH denaturation of insulin.

Figures 9A and 9B are graphic illustrations of the reversibility of the circular dichroism spectrum of  $\alpha$ -interferon.

Figure 10 is a graphic illustration of the circular dichroism spectrum of  $\alpha$ -interferon.

Figure 11 is a graphic illustration of intrinsic tryptophan fluorescence of  $\alpha$ -interferon and a complexing perturbant.

Figure 12 is a graphic illustration of serum levels of  $\alpha$ -interferon after oral administration of  $\alpha$ -interferon with and without complexing perturbant.

Figure 13 is a graphic illustration of the differential scanning calorimetry of  $\alpha$ -interferon and complexing perturbant.

5        Figures 14A and 14B are graphic illustrations of the reversibility of the transformation due to complexing perturbants.

Figure 15 is a graphic illustration of the effect of complexing perturbant on  $\alpha$ -interferon.

10        Figure 16 is a graphic illustration of serum levels of  $\alpha$ -interferon after oral administration of  $\alpha$ -interferon with and without complexing perturbant.

Figure 17 is a graphic illustration of the concentration effect of complexing perturbant on  $\alpha$ -interferon conformation.

Figure 18 is a graphic illustration of serum levels of  $\alpha$ -interferon after oral administration with and without complexing perturbant.

15        Figure 19 is a graphic illustration of the effect of complexing perturbant on  $\alpha$ -interferon.

Figure 20 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

20        Figure 21 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figure 22 is a graphic illustration of the effects of complexing perturbants on  $\alpha$ -interferon.

Figure 23 is a graphic illustration of the effect of the concentration of complexing perturbants on  $\alpha$ -interferon.

25        Figure 24 is a graphic illustration of the Isothermal Titration Calorimetry of  $\alpha$ -interferon and complexing perturbant.

Figure 25 is a graphic illustration of serum levels of  $\alpha$ -interferon after oral administration with complexing perturbants.

30        Figure 26 is a graphic illustration of the *in vivo* pharmacokinetics of recombinant human growth hormone mixed with complexing perturbants.

Figure 27 is a graphic illustration of pancreatic inhibition assay with  $\alpha$ -interferon and complexing perturbants.

Figure 28 is a graphic illustration of the effect of DSC of heparin at pH 5.0.

Figure 29 is a graphic illustration of the degree of retardation vs. peak APTT values from *in vivo* dosing experiments with heparin.

5 Figure 30 is a graphic illustration of clotting time in rats after oral administration of heparin with and without complexing perturbants.

### **DETAILED DESCRIPTION OF THE INVENTION**

All biological organisms can be described as being comprised of aqueous compartments separated from one another by cell membranes or lipid bilayers. Active agents, and particularly pharmacologic or therapeutic active agents, have one solubility value in an aqueous environment and an entirely different solubility value in a hydrophobic environment. Typically, delivery of an active agent from the site of administration to the target site, such as a site of pathology, requires passing the active agent through cell membranes or lipid bilayers in which the solubility of the active agent will vary. Additionally, oral delivery of active agent requires the ability to resist enzymatic degradation, pH differentials, and the like. These barriers result in significant irreversible partial, or in some instances total, loss of the active agent or its biological activity between the site of administration and the target. Consequently, the quantity of active agent that is required to elicit a proper response, such as a therapeutic response, may not reach the target. Therefore, active agents require some assistance in reaching and then in crossing these membranes or lipid bilayers.

The present invention effects active agent delivery by creating a reversibly non-covalently complexed supramolecule from the active agent and complexing perturbant. As a result, the three-dimensional structure or conformation of the active agent is changed, but the chemical composition of the active agent molecule is not altered. This alteration in structure (but not composition) provides the active agent with the appropriate solubility (log P) to cross or penetrate the membrane or lipid bilayer and to resist enzymatic degradation and the like. Crossing refers to transport from one side of the cell membrane or lipid bilayer to the opposite side (i.e. from the outside or exterior to the inside or interior of a cell and/or visa versa), whether the cell membrane

or lipid bilayer is actually penetrated or not. Additionally, the perturbed intermediate state of the active agent or the supramolecular complex itself can be used as a template for the preparation of mimetics which would, accordingly, be transportable across a cell membrane or a lipid bilayer. After crossing the cell membrane or lipid bilayer, an active agent has biological activity and bioavailability, either by restoration to the native state or by retaining biological activity or bioavailability acquired in the intermediate state. The mimetic acts similarly after crossing the cell membrane or lipid bilayer.

### 10      Active Agents

The native conformational state of an active agent is typically described as the three dimensional state with the lowest free energy ( $\Delta G$ ). It is the state in which the active agent typically possesses the full complement of activity ascribed to the agent, such as the full complement of biological activity ascribed to a biologically active agent.

The denatured conformational state is the state in which the active agent has no secondary or tertiary structure.

Intermediate conformational states exist between the native and denatured states. A particular active agent may have one or more intermediate states. The intermediate state achieved by the present invention is structurally and energetically distinct from both the native and denatured states. Active agents useful in the present invention must be transformable from their native conformational state to a transportable intermediate conformational state and back to their native state, i.e. reversibly transformable, so that when the active agent reaches its target, such as when an orally delivered drug reaches the circulatory system, the active agent retains, regains, or acquires a biologically, pharmacologically, or therapeutically significant complement of its desired biological activity. Preferably the  $\Delta G$  of the intermediate state ranges from about -20 Kcal/mole to about 20 Kcal/mole, and most preferably, it ranges from about -10 Kcal/mole to about 10 Kcal/mole.

For example in the case of a protein, the intermediate state has significant secondary structure, significant compactness due to the presence of a sizable hydrophobic core, and a tertiary structure reminiscent of the native fold

but without necessarily exhibiting the packing of the native state. The difference in free energy ( $\Delta G$ ) between the intermediate state and the native state is relatively small. Hence, the equilibrium constant between the native and the transportable, reversible intermediate state(s) is close to unity (depending upon experimental conditions). Intermediate states can be confirmed by, for example, differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), native gradient gels, NMR, fluorescence, and the like.

Without being bound by any theory, applicants believe that the physical chemistry of the intermediate state can be understood by the following explanation relating to proteinaceous active agents. Proteins can exist in stable intermediate conformations that are structurally and energetically distinct from either the native state or the denatured state. The inherent stability of any conformation(s) of any protein is reflected in the Gibbs free energy of the conformation(s). The Gibbs free energy for any state of a monomeric protein is described thermodynamically by the following relationship:

$$\Delta G^{\circ} = \Delta H^{\circ}(T_R) - T\Delta S^{\circ}(T_R) + \Delta C_p^{\circ} ( (T-T_R) - T \ln(T/T_R) ) \quad (1)$$

where  $T$  is the temperature,  $T_R$  is a reference temperature,  $\Delta H^{\circ}(T_R)$  and  $T\Delta S^{\circ}(T_R)$  are the relative enthalpy and entropy of this state at the reference temperature, and  $\Delta C_p^{\circ}$  is the relative heat capacity of this state. It is convenient to choose the native state as the reference state to express all relative thermodynamic parameters.

The sum of the statistical weights of all states accessible to the protein is defined as the partition function  $Q$ :

$$Q = \sum_{i=0}^n e^{-\Delta G_i/RT} \quad (2)$$

Equation 2 can also be written as

$$Q = 1 + \sum_{i=1}^{n-1} e^{-\Delta G_i / RT} + e^{-\Delta G_n / RT} \quad (3)$$

5

where the second term includes all the intermediates that become populated during the transition. The first and last terms of equation (3) are the statistical weights of the native and denatured states, respectively. Under most conditions, protein structure could be approximated by a two-state transition

10 function:

$$Q \approx 1 + e^{-\Delta G / RT} \quad (4)$$

See, Tanford, C., *Advances in Protein Chemistry*, 1968, 23, 2-95. Conformations of proteins that are intermediate between the native state and the denatured state can be detected by, for example, NMR, calorimetry, and fluorescence. Dill, K.A., Shortle, D., *Annu. Rev. Biochem.* 60, 1991, 795-825.

All thermodynamic parameters can be expressed in terms of the partition function. Specifically the population of molecules in state *i* is given in equation (5)

$$\Pi_i = \frac{e^{-\Delta G_i / RT}}{Q} \quad (5)$$

25

Therefore, measurement of the appropriate terms in equation (1) that would allow for the calculation of the Gibbs free energy would determine the extent

to which any intermediate state(s) is populated to any significant degree under defined experimental conditions. This in turn indicates the role that these intermediate state(s) play in drug delivery. The more populated the intermediate state, the more efficient the delivery.

5

Active agents suitable for use in the present invention include biologically active agents and chemically active agents, including, but not limited to, fragrances, as well as other active agents such as, for example, cosmetics.

10

Biologically active agents include, but are not limited to, pesticides, pharmacological agents, and therapeutic agents. For example, biologically active agents suitable for use in the present invention include, but are not limited to, peptides, and particularly small peptides; hormones, and particularly hormones which by themselves do not or only pass slowly through the gastro-intestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes in the gastro-intestinal tract; polysaccharides, and particularly mixtures of muco-polysaccharides; carbohydrates; lipids; or any combination thereof. Further examples include, but are not limited to, human growth hormones; bovine growth hormones; growth releasing hormones; interferons; interleukin-1; insulin; heparin, and particularly low molecular weight heparin; calcitonin; erythropoietin; atrial natriuretic factor; antigens; monoclonal antibodies; somatostatin; adrenocorticotropin, gonadotropin releasing hormone; oxytocin; vasopressin; cromolyn sodium (sodium or disodium chromoglycate); vancomycin; desferrioxamine (DFO); anti-microbials, including, but not limited to anti-fungal agents; or any combination thereof.

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25

The methods and compositions of the present invention may combine one or more active agents.

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### Perturbants

Perturbants serve two purposes in the present invention. In a first embodiment, the active agent is contacted with a perturbant which reversibly transforms the active agent from the native state to the

intermediate transportable state. The perturbant non-covalently complexes with the active agent to form a supramolecular complex which can permeate or cross cell membranes and lipid bilayers. This supramolecular complex can be used as a template for the design of a mimetic or can be used as a delivery composition itself. The perturbant, in effect, fixes the active agent in the transportable intermediate state. The perturbant can be released from the supramolecular complex, such as by dilution in the circulatory system, so that the active agent can return to the native state. Preferably, these perturbants have at least one hydrophilic (i.e. readily soluble in water, such as for example, a carboxylate group) and at least one hydrophobic moiety (i.e. readily soluble in an organic solvent such as, for example, a benzene group), and have a molecular weight ranging from about 150 to about 600 daltons and most preferably from about 200 to about 500 daltons.

Complexing perturbant compounds include, but are not limited to proteinoids including linear, non-linear, and cyclic proteinoids; modified (acylated or sulfonated) amino acids, poly amino acids, and peptides; modified amino acid, poly amino acid, or peptide derivatives (ketones or aldehydes); diketopiperazine/amino acid constructs; carboxylic acids; and various other perturbants discussed below.

Again without being bound by any theory, applicant believes that the non-covalent complexing may be effected by intermolecular forces including but not limited to, hydrogen bonding, hydrophilic interactions, electrostatic interactions, and Van der Waals interactions. For any given active agent/perturbant supramolecular complex, there will exist some combination of the aforementioned forces that maintain the association.

The association constant  $K_a$  between the perturbant and the active agent can be defined according to equation (6)

$$K_a = e^{-\Delta G/RT} \quad (6)$$

30

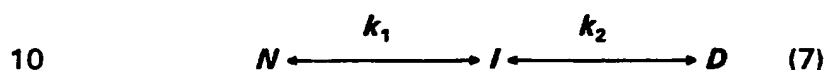
The dissociation constant  $K_d$  is the reciprocal of  $K_a$ . Thus measurement of the association constants between perturbant and active



agent at a defined temperature will yield data on the molar Gibbs free energy which allows for the determination of the associated enthalpic and entropic effects. Experimentally these measurements can be made, for example, using NMR, fluorescence or calorimetry.

5 This hypothesis can be illustrated with proteins in the following manner:

Protein unfolding can be described according to the equilibrium that exists between its various conformational states, e.g.



where N is the native state, I is the intermediate state(s), D is the denatured state, and  $k_1$  and  $k_2$  are the respective rate constants.  $K_1$  and  $K_2$  are the  
15 respective equilibrium constants. Accordingly,

$$Q = \sum_{i=0}^n e^{-\Delta G_i/RT} \quad (2)$$

20 
$$= 1 + e^{-\Delta G_1/RT} + e^{-\Delta G_2/RT}$$

$$= 1 + K_1 + K_2 \quad (8)$$

$$= 1 + k_1 + k_1 k_2 \quad (9)$$

This suggests that increasing the partition function of the  
25 intermediate state(s) should have a positive impact on the ability to deliver the active agent, i.e.

(10)

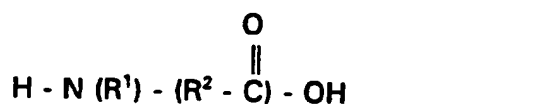
Because complexing must be reversible, the complexing of the perturbant with the active agent, as measured by the  $K_d$ , must be strong enough to insure delivery of the drug either to the systemic circulation and/or to the target(s), but not so strong so that disengagement of the perturbant will not occur in a timely manner to allow the active agent to renature if necessary to produce the desired effect(s).

In a second embodiment, perturbants reversibly transform the active agent to the intermediate state so that the conformation of that state can be used as a template for the preparation of mimetics. Perturbants for this purpose need not, but may, complex with the active agent. Therefore, in addition to the complexing perturbants discussed above, perturbants that change the pH of the active agent or its environment, such as for example, strong acids or strong bases; detergents; perturbants that change the ionic strength of the active agent or its environment; other agents such as for example, guanidine hydrochloride; and temperature can be used to transform the active agent. Either the supramolecular complex or the reversible intermediate state can be used as a template for mimetic design.

#### Complexing Perturbants

Amino acids are the basic materials used to prepare many of the complexing perturbants useful in the present invention. An amino acid is any carboxylic acid having at least one free amine group and includes naturally occurring and synthetic amino acids. The preferred amino acids for use in the present invention are  $\alpha$ -amino acids, and most preferably are naturally occurring  $\alpha$ -amino acids. Many amino acids and amino acid esters are readily available from a number of commercial sources such as Aldrich Chemical Co. (Milwaukee, WI, USA); Sigma Chemical Co. (St. Louis, MO, USA); and Fluka Chemical Corp. (Ronkonkoma, NY, USA).

Representative, but not limiting, amino acids suitable for use in the present invention are generally of the formula



5 wherein:  $\text{R}^1$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl, or  $\text{C}_2$ - $\text{C}_4$  alkenyl;

$\text{R}^2$  is  $\text{C}_1$ - $\text{C}_{24}$  alkyl,  $\text{C}_2$ - $\text{C}_{24}$  alkenyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkenyl, phenyl, naphthyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl), or naphthyl ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl);

$\text{R}^2$  being optionally substituted with  $\text{C}_1$ - $\text{C}_4$  alkyl,  $\text{C}_2$ - $\text{C}_4$  alkenyl,  $\text{C}_1$ - $\text{C}_4$  alkoxy, -OH, -SH, - $\text{CO}_2\text{R}^3$ ,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkenyl, heterocycle having 3-10 ring atoms wherein the hetero atom is one or more of N, O, S, or any combination thereof, aryl, ( $\text{C}_1$ - $\text{C}_{10}$  alk)aryl, ar( $\text{C}_1$ - $\text{C}_{10}$  alkyl) or any combination thereof;

$\text{R}^2$  being optionally interrupted by oxygen, nitrogen, sulfur, or any combination thereof; and

20  $\text{R}^3$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl, or  $\text{C}_2$ - $\text{C}_4$  alkenyl.

The preferred naturally occurring amino acids for use in the present invention as amino acids or components of a peptide are alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, 25 phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, hydroxy proline,  $\gamma$ -carboxyglutamate, phenylglycine, or O-phosphoserine. The preferred amino acids are arginine, leucine, lysine, phenylalanine, tyrosine, tryptophan, valine, and phenylglycine.

The preferred non-naturally occurring amino acids for use in the 30 present invention are  $\beta$ -alanine,  $\alpha$ -amino butyric acid,  $\gamma$ -amino butyric acid,  $\gamma$ -(aminophenyl) butyric acid,  $\alpha$ -amino isobutyric acid, citrulline,  $\epsilon$ -amino caproic acid, 7-amino heptanoic acid,  $\beta$ -aspartic acid, aminobenzoic acid, aminophenyl acetic acid, aminophenyl butyric acid,  $\gamma$ -glutamic acid, cysteine (ACM),  $\epsilon$ -lysine,  $\epsilon$ -lysine (A-Fmoc), methionine sulfone, norleucine, norvaline,

ornithine, D-ornithine, p-nitro-phenylalanine, hydroxy proline, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, and thioproline.

Poly amino acids are either peptides or two or more amino acids linked by a bond formed by other groups which can be linked, *e.g.*, an ester, anhydride or an anhydride linkage. Special mention is made of non-naturally occurring poly amino acids and particularly non-naturally occurring heteropoly amino acids, i.e. of mixed amino acids.

Peptides are two or more amino acids joined by a peptide bond. Peptides can vary in length from di-peptides with two amino acids to polypeptides with several hundred amino acids. See, Walker, Chambers Biological Dictionary, Cambridge, England: Chambers Cambridge, 1989, page 215. Special mention is made of non-naturally occurring peptides and particularly non-naturally occurring peptides of mixed amino acids. Special mention is also made of di-peptides tri-peptides, tetra-peptides, and pentapeptides, and particularly, the preferred peptides are di-peptides and tri-peptides. Peptides can be homo- or hetero- peptides and can include natural amino acids, synthetic amino acids, or any combination thereof.

#### Proteinoid Complexing Perturbants

Proteinoids are artificial polymers of amino acids. The proteinoids preferably are prepared from mixtures of amino acids. Preferred proteinoids are condensation polymers, and most preferably, are thermal condensation polymers. These polymers may be directed or random polymers. Proteinoids can be linear, branched, or cyclical, and certain proteinoids can be units of other linear, branched, or cyclical proteinoids.

Special mention is made of diketopiperazines. Diketopiperazines are six member ring compounds. The ring includes two nitrogen atoms and is substituted at two carbons with two oxygen atoms. Preferably, the carbonyl groups are at the 1 and 4 ring positions. These rings can be optionally, and most often are, further substituted.

Diketopiperazine ring systems may be generated during thermal polymerization or condensation of amino acids or amino acid derivatives. (Gyore, J; Ecat M. *Proceedings Fourth ICTA (Thermal Analysis)*, 1974, 2,

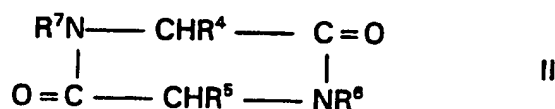
387-394 (1974)). These six membered ring systems were presumably generated by intra-molecular cyclization of the dimer prior to further chain growth or directly from a linear peptide (Reddy, A.V., *Int. J. Peptide Protein Res.*, 40, 472-476 (1992); Mazurov, A.A. et al., *Int. J. Peptide Protein Res.*, 5 42, 14-19 (1993)).

Diketopiperazines can also be formed by cyclodimerization of amino acid ester derivatives as described by Katchalski et al., *J. Amer. Chem. Soc.*, 68, 879-880 (1946), by cyclization of dipeptide ester derivatives, or by thermal dehydration of amino acid derivatives and high boiling solvents as described by Kopple et al., *J. Org. Chem.*, 33 (2), 862-864 (1968).

In a typical synthesis of a diketopiperazine, the COOH group(s) of an amino acid benzyl ester are activated in a first step to yield a protected ester. The amine is deprotected and cyclized via dimerization in a second step, providing a diketopiperazine di-ester. Finally, the COOH group(s) are deprotected to provide the diketopiperazine.

Diketopiperazines typically are formed from  $\alpha$ -amino acids. Preferably, the  $\alpha$ -amino acids of which the diketopiperazines are derived are glutamic acid, aspartic acid, tyrosine, phenylalanine, and optical isomers of any of the foregoing.

Special mention is made of diketopiperazines of the formula

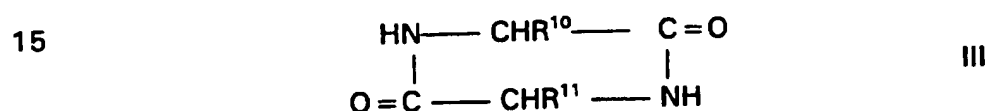


wherein  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently are hydrogen,  $\text{C}_1$ - $\text{C}_{24}$  alkyl,  $\text{C}_1$ - $\text{C}_{24}$  alkenyl, phenyl, naphthyl,  $(\text{C}_1$ - $\text{C}_{10}$  alkyl)phenyl,  $(\text{C}_1$ - $\text{C}_{10}$  alkenyl)phenyl,  $(\text{C}_1$ - $\text{C}_{10}$  alkyl)naphthyl,  $(\text{C}_1$ - $\text{C}_{10}$  alkenyl)naphthyl, phenyl  $(\text{C}_1$ - $\text{C}_{10}$  alkyl), phenyl  $(\text{C}_1$ - $\text{C}_{10}$  alkenyl), naphthyl  $(\text{C}_1$ - $\text{C}_{10}$  alkyl), and naphthyl  $(\text{C}_1$ - $\text{C}_{10}$  alkenyl); any of  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently may optionally be substituted with  $\text{C}_1$ - $\text{C}_4$  alkyl,  $\text{C}_1$ - $\text{C}_4$  alkenyl,  $\text{C}_1$ - $\text{C}_4$  alkoxy, -OH, -SH, and  $-\text{CO}_2\text{R}^8$  or any combination thereof;  $\text{R}^8$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl or  $\text{C}_1$ - $\text{C}_4$  alkenyl; and any of  $\text{R}^4$ ,  $\text{R}^5$ ,  $\text{R}^6$ , and  $\text{R}^7$  independently may optionally be interrupted by oxygen, nitrogen, sulfur, or any combination thereof.

The phenyl or naphthyl groups may optionally be substituted. Suitable, but non-limiting, examples of substituents are C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>1</sub>-C<sub>6</sub> alkenyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, -OH, -SH, or CO<sub>2</sub>R<sup>9</sup> wherein R<sup>9</sup> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, or C<sub>1</sub>-C<sub>6</sub> alkenyl.

- 5 Preferably, R<sup>6</sup> and R<sup>7</sup> independently are hydrogen, C<sub>1</sub>-C<sub>4</sub> alkyl or C<sub>1</sub>-C<sub>4</sub> alkenyl. Special mention is made of diketopiperazines which are preferred complexing perturbants. These diketopiperazines include the unsubstituted diketopiperazine in which R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, and R<sup>7</sup> are hydrogen, and diketopiperazines which are substituted at one or both of the nitrogen atoms  
10 in the ring, i.e. mono or di-N-substituted. Special mention is made of the N-substituted diketopiperazine wherein one or both of the nitrogen atoms is substituted with a methyl group.

Special mention is also made of diketopiperazines of the formula



- 20 wherein R<sup>10</sup> and R<sup>11</sup> independently are hydrogen, C<sub>1</sub>-C<sub>24</sub> alkyl, C<sub>1</sub>-C<sub>24</sub> alkenyl, phenyl, naphthyl, (C<sub>1</sub>-C<sub>10</sub> alkyl)phenyl, (C<sub>1</sub>-C<sub>10</sub> alkenyl)phenyl, (C<sub>1</sub>-C<sub>10</sub> alkyl)naphthyl, (C<sub>1</sub>-C<sub>10</sub> alkenyl)naphthyl, phenyl (C<sub>1</sub>-C<sub>10</sub> alkyl), phenyl(C<sub>1</sub>-C<sub>10</sub> alkenyl), naphthyl (C<sub>1</sub>-C<sub>10</sub> alkyl), and naphthyl (C<sub>1</sub>-C<sub>10</sub> alkenyl); but both R<sup>10</sup> and R<sup>11</sup> can not be hydrogen; either or both R<sup>10</sup> or R<sup>11</sup> independently may  
25 optionally be substituted with C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkenyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, -OH, -SH, and -CO<sub>2</sub>R<sup>12</sup> or any combination thereof; R<sup>12</sup> is hydrogen, C<sub>1</sub>-C<sub>4</sub> alkyl or C<sub>1</sub>-C<sub>4</sub> alkenyl; and either or both R<sup>10</sup> and R<sup>11</sup> independently may optionally be interrupted by oxygen, nitrogen, sulfur, or any combination thereof.

- The phenyl or naphthyl groups may optionally be substituted.  
30 Suitable, but non-limiting, examples of substituents are C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>1</sub>-C<sub>6</sub> alkenyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, -OH, -SH, or CO<sub>2</sub>R<sup>13</sup> wherein R<sup>13</sup> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, or C<sub>1</sub>-C<sub>6</sub> alkenyl. When one of R<sup>10</sup> or R<sup>11</sup> is hydrogen, the diketopiperazine is mono-carbon-(C)-substituted. When neither R<sup>10</sup> nor R<sup>11</sup> is hydrogen, the diketopiperazine is di-carbon-(C)-substituted.

Preferably,  $R^{10}$ ,  $R^{11}$ , or both  $R^{10}$  and  $R^{11}$ , contain at least one functional group, a functional group being a non-hydrocarbon portion responsible for characteristic reactions of the molecule. Simple functional groups are heteroatoms including, but not limited to halogens, oxygen, sulfur, nitrogen, and the like, attached to, the carbon of an alkyl group by a single or multiple bond. Other functional groups include, but are not limited to, for example, hydroxyl groups, carboxyl groups, amide groups, amine groups, substituted amine groups, and the like.

Preferred diketopiperazines are those which are substituted at one or two of the carbons of the ring with a functional group that includes at least one carboxyl functionality.

#### Amino Acid(s)/Diketopiperazine Complexing Perturbants

Diketopiperazines may also be polymerized with additional amino acids to form constructs of at least one amino acid or an ester or an amide thereof and at least one diketopiperazine, preferably covalently bonded to one another.

When the diketopiperazine is polymerized with additional amino acids, one or more of the R groups must contain at least one functional group, a functional group being a non-hydrocarbon portion responsible for characteristic reactions of the molecule. Simple functional groups are heteroatoms including, but not limited to halogens, oxygen, sulfur, nitrogen, and the like, attached to, the carbon of an alkyl group by a single or multiple bond. Other functional groups include, but are not limited to, for example, hydroxyl groups, carboxyl groups, amide groups, amine groups, substituted amine groups, and the like.

Special mention is also made of diketopiperazines which are preferred components of the amino acids/diketopiperazine perturbants of the present invention. Such preferred diketopiperazines are those which are substituted at one or two of the carbons of the ring and preferably are substituted with a functional group that includes at least one carboxyl functionality.

Most preferably, the diketopiperazines in the amino acids/diketopiperazine perturbants are prepared from trifunctional amino acids such as L-glutamic acid and L-aspartic acid which cyclize to form diketopiperazines.

5           The diketopiperazines can generate a bis-carboxylic acid platform which can be further condensed with other amino acids to form the perturbant. Typically, the diketopiperazine will react and covalently bond with one or more of the amino acids through the functional group(s) of the R groups of the diketopiperazines. These unique systems, because of the cis-  
10       geometry imparted by the chiral components of the diketopiperazine ring (Lannom, H.K. et al., *Int. J. Peptide Protein Res.*, 28, 67-78 (1986)), provide an opportunity to systematically alter the structure of the terminal amino acids while holding the orientation between them fixed relative to non-cyclic  
15       analogues (Fusaoka et al., *Int. J. Peptide Protein Res.*, 34, 104-110 (1989); Ogura, H. et al., *Chem. Pharma. Bull.*, 23, 2474-2477 (1975). See also, Lee, B.H. et al. *J. Org. Chem.*, 49, 2418-2423 (1984); Buyle, R., *Helv. Chim. Acta*, 49, 1425, 1429 (1966). Other methods of polymerization known to those skilled in the art may lend themselves to amino acid/diketopiperazine polymerization as well.

20           The amino acids/diketopiperazine perturbants may include one or more of the same or different amino acids as well as one or more of the same or different diketopiperazines as described above.

          Ester and amide derivatives of these amino acids/diketopiperazine perturbants are also useful in the present invention.

25

#### Modified Amino Acid Complexing Perturbants

Modified amino acids, poly amino acids or peptides are either acylated or sulfonated and include amino acid amides and sulfonamides.

30

#### Acylated Amino Acid Complexing Perturbants

Special mention is made of acylated amino acids having the formula





IV

wherein Ar is a substituted or unsubstituted phenyl or naphthyl;

5 Y is  $-\overset{\text{O}}{\parallel}{\text{C}}-$ ,  $\text{R}^{14}$  has the formula  $-\text{N}(\text{R}^{16})-\text{R}^{15}-\overset{\text{O}}{\parallel}{\text{C}}-$ , wherein:

$\text{R}^{15}$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl,  $\text{C}_1$  to  $\text{C}_{24}$  alkenyl, phenyl, naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl),  
 10 naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) and naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl);

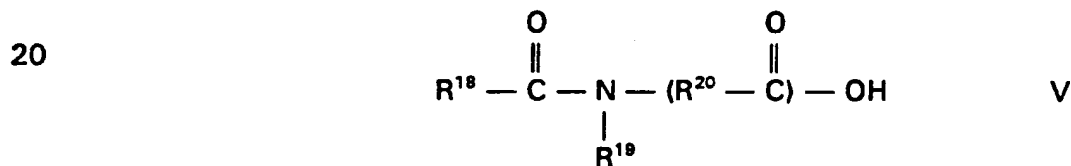
$\text{R}^{15}$  is optionally substituted with  $\text{C}_1$  to  $\text{C}_4$  alkyl,  $\text{C}_1$  to  $\text{C}_4$  alkenyl,  $\text{C}_1$  to  $\text{C}_4$  alkoxy, -OH, -SH and  $-\text{CO}_2\text{R}^5$ , cycloalkyl, cycloalkenyl, heterocyclic alkyl, alkaryl, heteroaryl, heteroalkaryl, or any combination thereof;

$\text{R}^{17}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl;

15  $\text{R}^{15}$  is optionally interrupted by oxygen, nitrogen, sulfur or any combination thereof; and

$\text{R}^{16}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl.

Special mention is also made of those having the formula



25 wherein:  $\text{R}^{18}$  is (i)  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl, optionally substituted with  $\text{C}_1$ - $\text{C}_7$  alkyl,  $\text{C}_2$ - $\text{C}_7$  alkenyl,  $\text{C}_1$ - $\text{C}_7$  alkoxy, hydroxy, phenyl, phenoxy or  $-\text{CO}_2\text{R}^{21}$ , wherein  $\text{R}^1$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl, or  $\text{C}_2$ - $\text{C}_4$  alkenyl; or

(ii)  $\text{C}_1$ - $\text{C}_6$  alkyl substituted with  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl;

$\text{R}^{19}$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  alkyl, or  $\text{C}_2$ - $\text{C}_4$  alkenyl;

30  $\text{R}^{20}$  is  $\text{C}_1$ - $\text{C}_{24}$  alkyl,  $\text{C}_2$ - $\text{C}_{24}$  alkenyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkenyl, phenyl, naphthyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$ - $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$ - $\text{C}_{10}$  alkyl) or naphthyl ( $\text{C}_2$ - $\text{C}_{10}$  alkenyl);

$\text{R}^{20}$  being optionally substituted with  $\text{C}_1$ - $\text{C}_4$  alkyl,  $\text{C}_2$ - $\text{C}_4$  alkenyl,

35  $\text{C}_1$ - $\text{C}_4$  alkoxy, -OH, -SH,  $-\text{CO}_2\text{R}^{22}$ ,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkyl,  $\text{C}_3$ - $\text{C}_{10}$  cycloalkenyl,

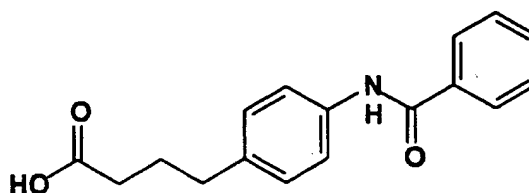
heterocycle having 3-10 ring atoms wherein the hetero atom is one or more of N, O, S or any combination thereof, aryl, (C<sub>1</sub>-C<sub>10</sub> alk)aryl, ar(C<sub>1</sub>-C<sub>10</sub> alkyl), or any combination thereof;

R<sup>20</sup> being optionally interrupted by oxygen, nitrogen, sulfur, or any combination thereof; and

R<sup>22</sup> is hydrogen, C<sub>1</sub>-C<sub>4</sub> alkyl, or C<sub>2</sub>-C<sub>4</sub> alkenyl.

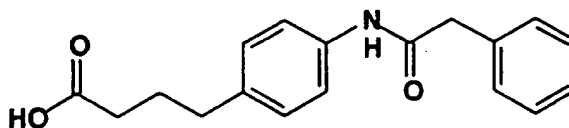
Some preferred acylated amino acids include salicyloyl phenylalanine, and the compounds having the formulas:

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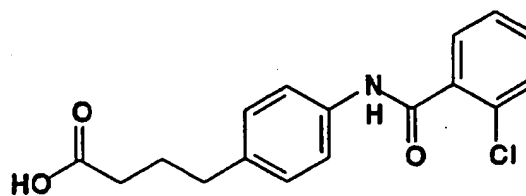
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VII

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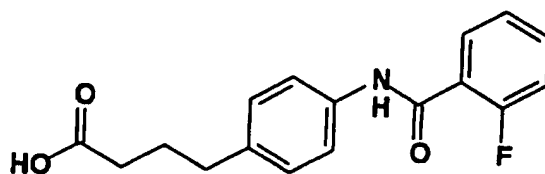


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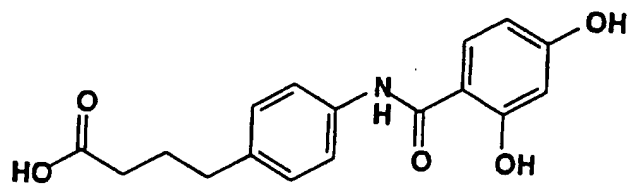
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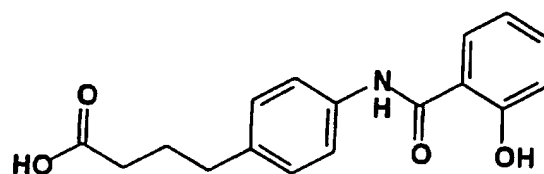
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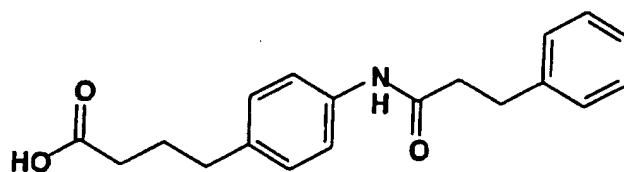
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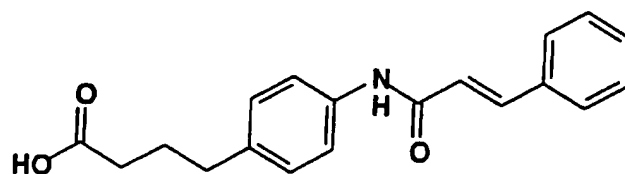
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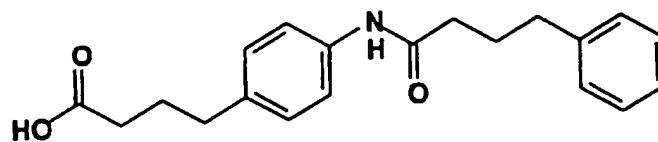
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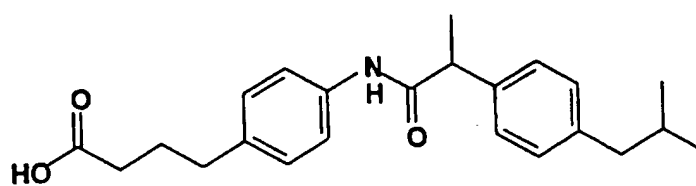
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XIV

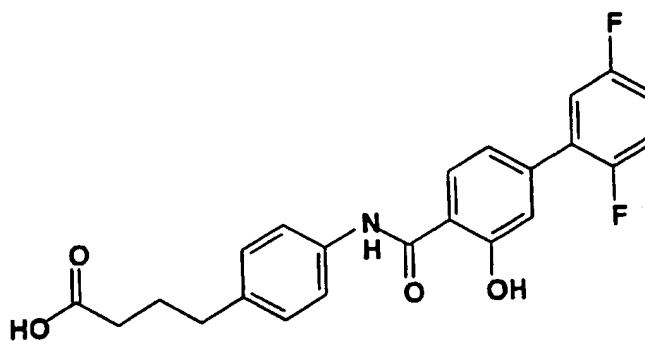
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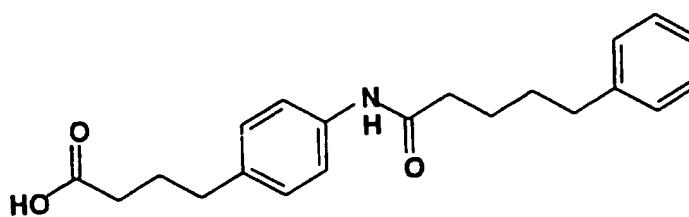
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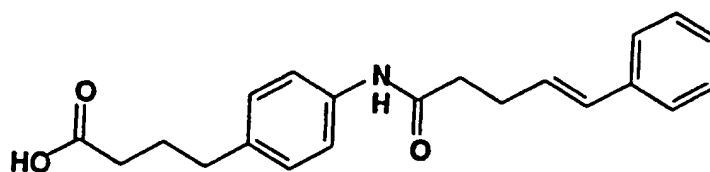
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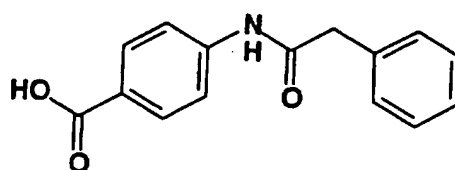
XVII

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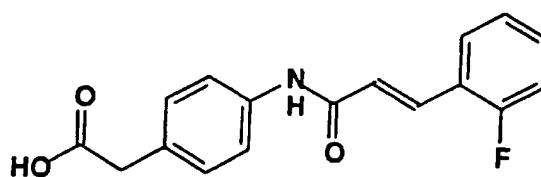
XVIII

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XIX

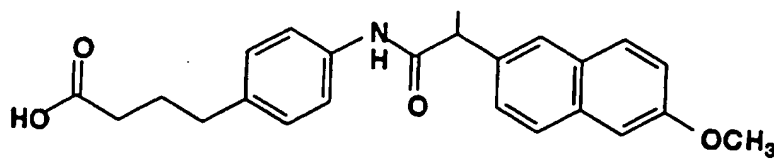
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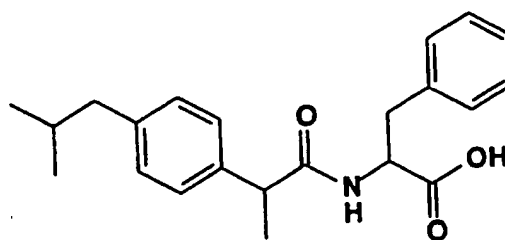


XXI

WO 96/12475

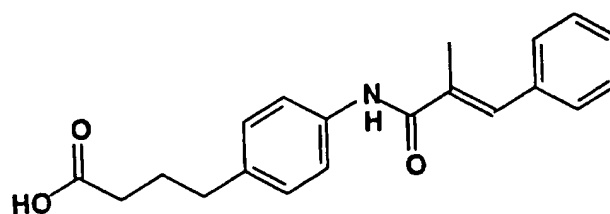
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XXII

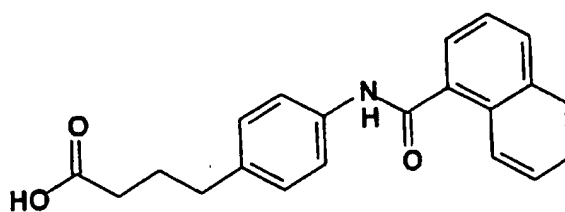
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XXIII

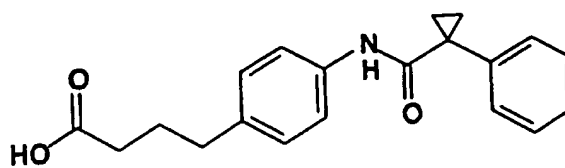
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XXIV

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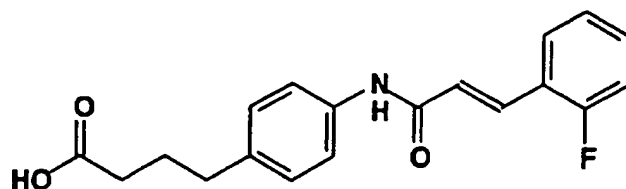


XXV

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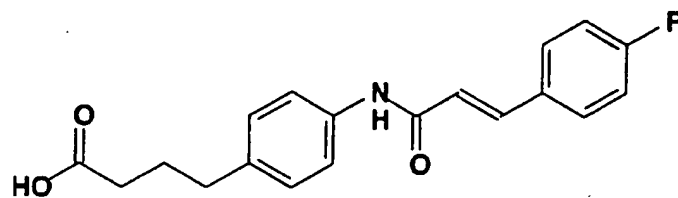
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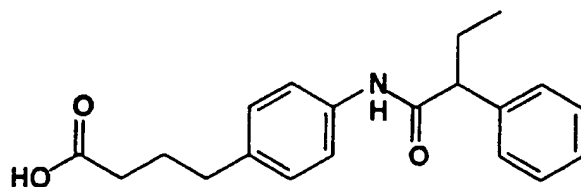
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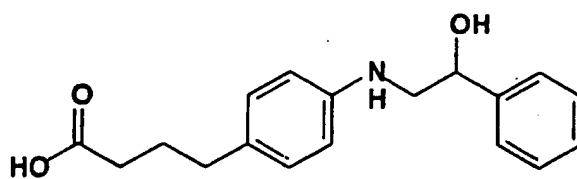
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XXVIII

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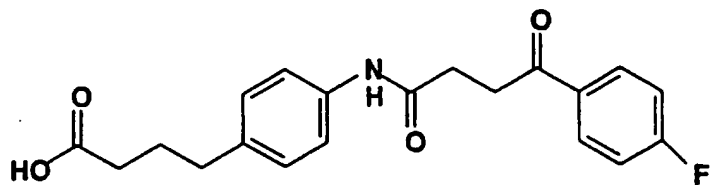


XIX

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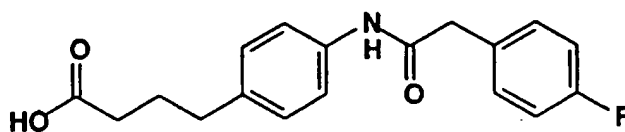
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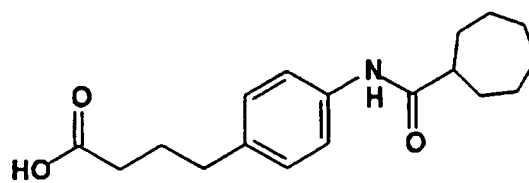
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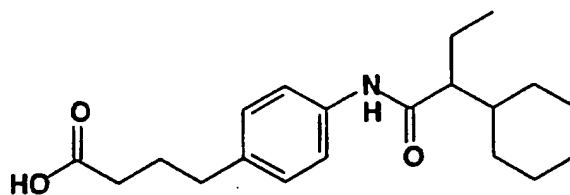
XXXI

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XXXII

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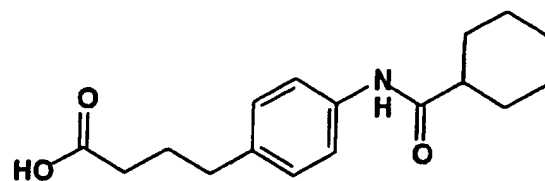
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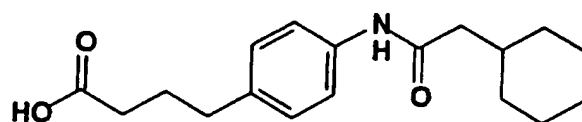
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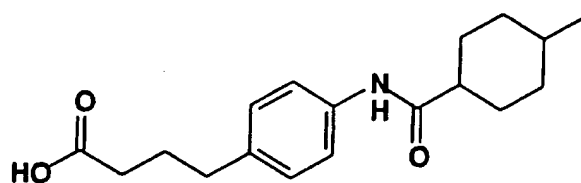
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XXXV

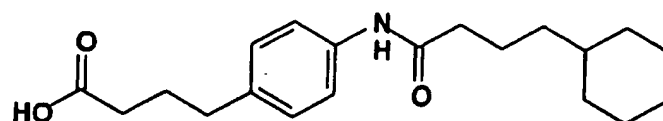
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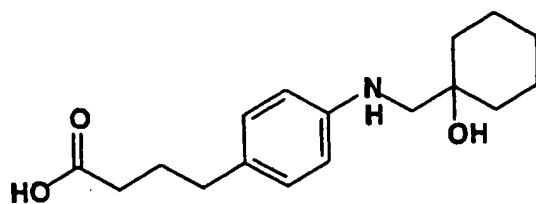
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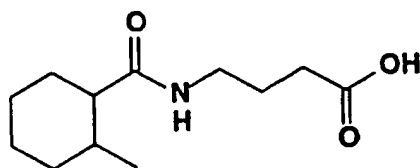
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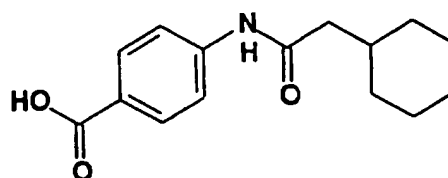
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XXXIX

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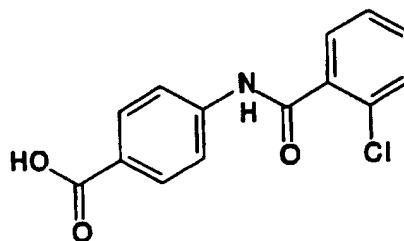


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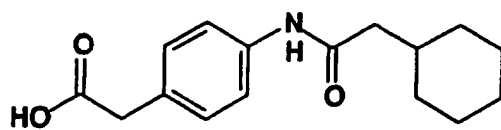
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XLI

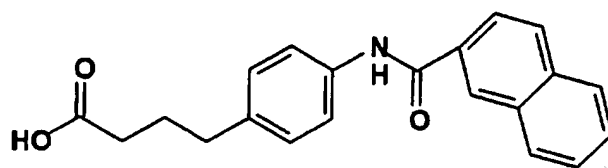
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XLII

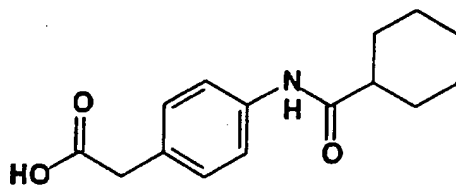
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XLIIA

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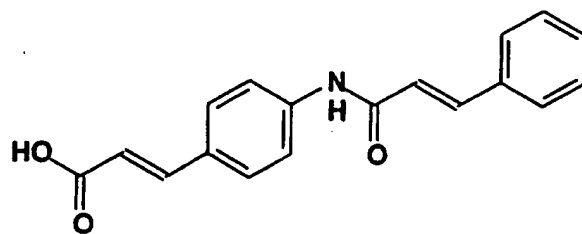
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XLIII

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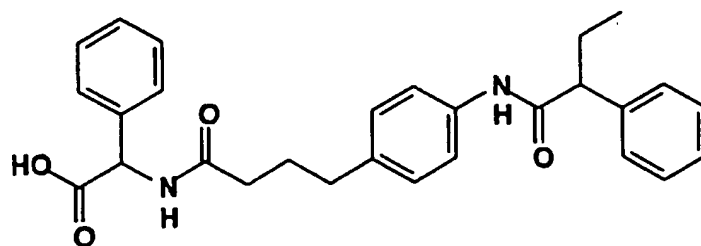


XLIV

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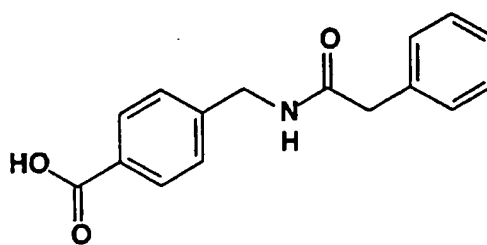
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XLV

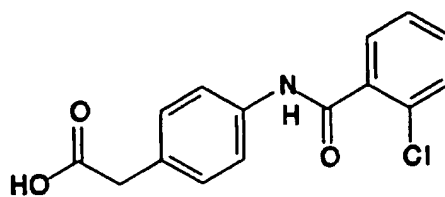
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XLVI

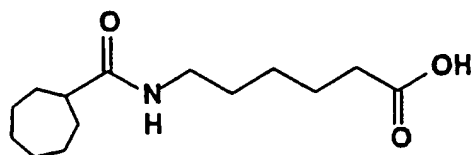
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XLVII

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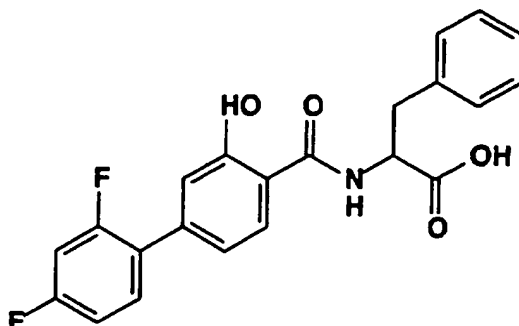


XLVIII

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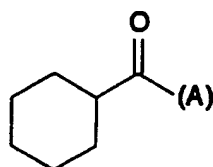


XLVIII

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Special mention is made of compounds having the formula:

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XLIX

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wherein A is Try, Leu, Arg, Trp, or Cit; and

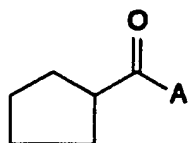
optionally wherein if A is Try, Arg, Trp or Cit; A is acylated at 2 or more functional groups.

Preferred compounds are those wherein A is Try; A is Tyr and is acylated at 2 functional groups; A is Leu; A is Arg; A is Arg and is acylated at 2 functional groups; A is Trp; A is Trp and is acylated at 2 functional groups; A is Cit; and A is Cit and is acylated at 2 functional groups.

25

Special mention is also made of compounds having the formula:

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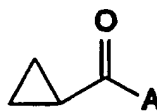


L

wherein A is Arg or Leu; and

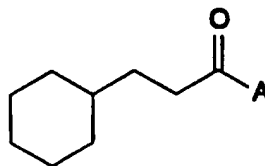
wherein if A is Arg, A is optionally acylated at 2 or more functional groups;

36



LI

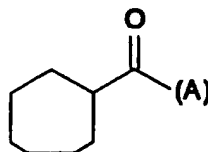
5 where A is Leu or phenylglycine;



LII

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wherein A is phenylglycine; and



LIII

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wherein A is phenylglycine.

20 Acylated amino acids may be prepared by reacting single amino acids, mixtures of two or more amino acids, or amino acid esters with an amine modifying agent which reacts with free amino moieties present in the amino acids to form amides.

25 Suitable, but non-limiting, examples of acylating agents useful in preparing acylated amino acids include

acid chloride acylating agents having the formula  $R^{23}-\overset{\overset{O}{\parallel}}{C}-X$  wherein:

30  $R^{23}$  an appropriate group for the modified amino acid being prepared, such as, but not limited to, alkyl, alkenyl, cycloalkyl, or aromatic, and particularly methyl, ethyl, cyclohexyl, cyclophenyl, phenyl, or benzyl, and X is a leaving group. Typical leaving groups include, but are not limited to, halogens such as chlorine, bromine and iodine.

Examples of the acylating agents include, but are not limited to, acyl halides including, but not limited to, acetyl chloride, propyl chloride, cyclohexanoyl chloride, cyclopentanoyl chloride, and cycloheptanoyl chloride, benzoyl chloride, hippuryl chloride and the like; and anhydrides, such as  
5 acetic anhydride, propyl anhydride, cyclohexanoic anhydride, benzoic anhydride, hippuric anhydride and the like. Preferred acylating agents include benzoyl chloride, hippuryl chloride, acetyl chloride, cyclohexanoyl chloride, cyclopentanoyl chloride, and cycloheptanoyl chloride.

The amine groups can also be modified by the reaction of a  
10 carboxylic acid with coupling agents such as the carbodiimide derivatives of amino acids, particularly hydrophilic amino acids such as phenylalanine, tryptophan, and tyrosine. Further examples include dicyclohexylcarbodiimide and the like.

If the amino acid is multifunctional, i.e. has more than one -OH, -  
15  $\text{NH}_2$  or -SH group, then it may optionally be acylated at one or more functional groups to form, for example, an ester, amide, or thioester linkage.

For example, in the preparation of many acylated amino acids, the amino acids are dissolved in an aqueous alkaline solution of a metal hydroxide, *e.g.*, sodium or potassium hydroxide and the acylating agent  
20 added. The reaction time can range from about 1 hour and about 4 hours, preferably about 2-2.5 hours. The temperature of the mixture is maintained at a temperature generally ranging between about 5°C and about 70°C, preferably between about 10°C and about 50°C. The amount of alkali employed per equivalent of  $\text{NH}_2$  groups in the amino acids generally ranges  
25 between about 1.25 moles and about 3 moles, and is preferably between about 1.5 moles and about 2.25 moles per equivalent of  $\text{NH}_2$ . The pH of the reaction solution generally ranges between about pH 8 and about pH 13, and is preferably between about pH 10 and about pH 12. The amount of amino modifying agent employed in relation to the quantity of amino acids is based  
30 on the moles of total free  $\text{NH}_2$  in the amino acids. In general, the amino modifying agent is employed in an amount ranging between about 0.5 and about 2.5 mole equivalents, preferably between about 0.75 and about 1.25 equivalents, per molar equivalent of total  $\text{NH}_2$  groups in the amino acids.

The modified amino acid formation reaction is quenched by adjusting the pH of the mixture with a suitable acid, *e.g.*, concentrated hydrochloric acid, until the pH reaches between about 2 and about 3. The mixture separates on standing at room temperature to form a transparent upper layer and a white or off-white precipitate. The upper layer is discarded, and modified amino acids are collected by filtration or decantation. The crude modified amino acids are then mixed with water. Insoluble materials are removed by filtration and the filtrate is dried in vacuo. The yield of modified amino acids generally ranges between about 30 and about 60%, and usually about 45%. The present invention also contemplates amino acids which have been modified by multiple acylation, *e.g.*, diacylation or triacylation.

If amino acid esters or amides are the starting materials, they are dissolved in a suitable organic solvent such as dimethylformamide or pyridine, are reacted with the amino modifying agent at a temperature ranging between about 5°C and about 70°C, preferably about 25°C, for a period ranging between about 7 and about 24 hours. The amount of amino modifying agents used relative to the amino acid esters are the same as described above for amino acids.

Thereafter, the reaction solvent is removed under negative pressure and optionally the ester or amide functionality can be removed by hydrolyzing the modified amino acid ester with a suitable alkaline solution, *e.g.*, 1N sodium hydroxide, at a temperature ranging between about 50°C and about 80°C, preferably about 70°C, for a period of time sufficient to hydrolyze off the ester group and form the modified amino acid having a free carboxyl group. The hydrolysis mixture is then cooled to room temperature and acidified, *e.g.*, with an aqueous 25% hydrochloric acid solution, to a pH ranging between about 2 and about 2.5. The modified amino acid precipitates out of solution and is recovered by conventional means such as filtration or decantation.

The modified amino acids may be purified by acid precipitation, recrystallization or by fractionation on solid column supports. Fractionation may be performed on a suitable solid column supports such as silica gel, alumina, using solvent mixtures such as acetic acid/butanol/water as the



mobile phase; reverse phase column supports using trifluoroacetic acid/acetonitrile mixtures as the mobile phase; and ion exchange chromatography using water as the mobile phase. The modified amino acids may also be purified by extraction with a lower alcohol such as methanol, butanol, or isopropanol to

5 remove impurities such as inorganic salts.

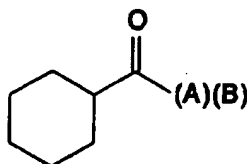
The modified amino acids generally are soluble in alkaline aqueous solution ( $\text{pH} \geq 9.0$ ); partially soluble in ethanol, n-butanol and 1:1 (v/v) toluene/ethanol solution and insoluble in neutral water. The alkali metal salts, *e.g.*, the sodium salt of the derivatized amino acids are generally

10 soluble in water at about a pH of 6-8.

In poly amino acids or peptides, one or more of the amino acids may be modified (acylated). Modified poly amino acids and peptides may include one or more acylated amino acid(s). Although linear modified poly amino acids and peptides will generally include only one acylated amino acid, other poly amino acid and peptide configurations can include more than one acylated amino acid. Poly amino acids and peptides can be polymerized with the acylated amino acid(s) or can be acylated after polymerization.

Special mention is made of the compound:

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LIV

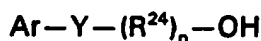
#### Sulfonated Amino Acid Complexing Perturbants

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Sulfonated modified amino acids, poly amino acids, and peptides are modified by sulfonating at least one free amine group with a sulfonating agent which reacts with at least one of the free amine groups present.

Special mention is made of compounds of the formula

30



LV

wherein Ar is a substituted or unsubstituted phenyl or naphthyl;

Y is  $-\text{SO}_2-$ ,  $\text{R}^{24}$  has the formula  $-\text{N}(\text{R}^{26})-\text{R}^{25}-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$ , wherein:

$\text{R}^{25}$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl,  $\text{C}_1$  to  $\text{C}_{24}$  alkenyl, phenyl, naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) phenyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) naphthyl, ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl) naphthyl, phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl), phenyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl), naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkyl) and naphthyl ( $\text{C}_1$  to  $\text{C}_{10}$  alkenyl);

$\text{R}^{25}$  is optionally substituted with  $\text{C}_1$  to  $\text{C}_4$  alkyl,  $\text{C}_1$  to  $\text{C}_4$  alkenyl,  $\text{C}_1$  to  $\text{C}_4$  alkoxy,  $-\text{OH}$ ,  $-\text{SH}$  and  $-\text{CO}_2\text{R}^{27}$  or any combination thereof;

$\text{R}^{27}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl;

$\text{R}^{25}$  is optionally interrupted by oxygen, nitrogen, sulfur or any combination thereof; and

$\text{R}^{26}$  is hydrogen,  $\text{C}_1$  to  $\text{C}_4$  alkyl or  $\text{C}_1$  to  $\text{C}_4$  alkenyl.

Suitable, but non-limiting, examples of sulfonating agents useful in preparing sulfonated amino acids include sulfonating agents having the formula  $\text{R}^{28}-\text{SO}_2-\text{X}$  wherein  $\text{R}^{28}$  is an appropriate group for the modified amino acid being prepared such as, but not limited to, alkyl, alkenyl, cycloalkyl, or aromatics and X is a leaving group as described above. One example of a sulfonating agent is benzene sulfonyl chloride.

Modified poly amino acids and peptides may include one or more sulfonated amino acid(s). Although linear modified poly amino acids and peptides used generally include only one sulfonated amino acid, other poly amino acid and peptide configurations can include more than one sulfonated amino acid. Poly amino acids and peptides can be polymerized with the sulfonated amino acid(s) or can be sulfonated after polymerization.

#### Modified Amino Acid Derivative Complexing Perturbants

Modified amino acid, polyamino acid, or peptide derivatives are amino acids, poly amino acids, or peptides which have had at least one acyl-terminus converted to an aldehyde or a ketone, and are acylated at at least one free amine group, with an acylating agent which reacts with at least one of the free amine groups present.

Amino acid, poly amino acid, or peptide derivatives can be readily prepared by reduction of amino acid esters or peptide esters with an appropriate reducing agent. For example, amino acid, poly amino acid, or peptide aldehydes can be prepared as described in an article by R. Chen *et al.*, *Biochemistry*, 1979, 18, 921-926. Amino acid, poly amino acid, or peptide ketones can be prepared by the procedure described in Organic Syntheses, Col. Vol. IV, Wiley, (1963), page 5. Acylation is discussed above.

For example, the derivatives may be prepared by reacting a single amino acid, poly amino acid, or peptide derivative or mixtures of two or more amino acid or peptide derivatives, with an acylating agent or an amine modifying agent which reacts with free amino moieties present in the derivatives to form amides. The amino acid, poly amino acid, or peptide can be modified and subsequently derivatized, derivatized and subsequently modified, or simultaneously modified and derivatized. Protecting groups may be used to avoid unwanted side reactions as would be known to those skilled in the art.

In modified poly amino acid or peptide derivative, one or more of the amino acid may be derivatized (an aldehyde or a ketone) and/or modified, (acylated) but there must be at least one derivative and at least one modification.

Special mention is made of the modified amino acid derivatives N-cyclohexanoyl-Phe aldehyde, N-acetyl-Phe-aldehyde, N-acetyl-Tyr ketone, N-acetyl-Lys ketone and N-acetyl-Leu ketone, and N-cyclohexanoyl phenyl-alanine aldehyde.

#### Carboxylic Acid Complexing Perturbants

Various carboxylic acids and salts of these carboxylic acids may be used as complexing perturbants. These carboxylic acids have the formula:



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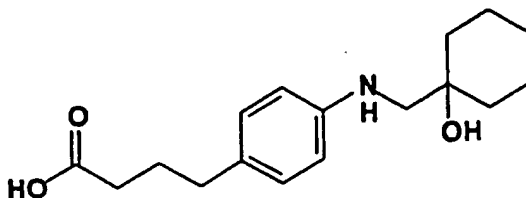
- wherein  $R^{29}$  is  $C_1$  to  $C_{24}$  alkyl,  $C_2$  to  $C_{24}$  alkenyl,  $C_3$  to  $C_{10}$  cycloalkyl,  $C_3$  to  $C_{10}$  cycl alkenyl, phenyl, naphthyl, ( $C_1$  to  $C_{10}$  alkyl)phenyl, ( $C_2$  to  $C_{10}$  alkenyl)phenyl, ( $C_1$  to  $C_{10}$  alkyl)naphthyl, ( $C_2$  to  $C_{10}$  alkenyl)naphthyl, phenyl( $C_1$  to  $C_{10}$  alkyl), phenyl( $C_2$  to  $C_{10}$  alkenyl),
- 5 naphthyl( $C_1$  to  $C_{10}$  alkyl) and naphthyl( $C_2$  to  $C_{10}$  alkenyl);
- $R^{29}$  being optionally substituted with  $C_1$  to  $C_{10}$  alkyl,  $C_2$  to  $C_{10}$  alkenyl,  $C_1$  to  $C_4$  alkoxy, -OH, -SH,  $-CO_2R^{30}$ ,  $C_3$  to  $C_{10}$  cycloalkyl,  $C_3$  to  $C_{10}$  cycloalkenyl, heterocyclic having 3-10 ring atoms wherein the hetero atom is one or more atoms of N, O, S or any combination thereof, aryl, ( $C_1$  to  $C_{10}$  alk)aryl, aryl( $C_1$  to  $C_{10}$ )alkyl, or any combination thereof;
- 10  $R^{29}$  being optionally interrupted by oxygen, nitrogen, sulfur, or any combination thereof; and

$R^{30}$  is hydrogen,  $C_1$  to  $C_4$  alkyl or  $C_2$  to  $C_4$  alkenyl.

- The preferred carboxylic acids are cyclohexanecarboxylic acid,
- 15 cyclopentanecarboxylic acid, cycloheptanecarboxylic acid, hexanoic acid, 3-cyclohexanepropanoic acid, methylcyclohexanecarboxylic acid, 1,2-cyclohexanedicarboxylic acid, 1,3-cyclohexanedicarboxylic acid, 1,4-cyclohexanedicarboxylic acid, 1-adamantanecarboxylic acid, phenylpropanoic acid, adipic acid, cyclohexanepentanoic acid, cyclohexanebutanoic acid,
- 20 pentylcyclohexanoic acid, 2-cyclopentanehexanoic acid, cyclohexane pentanoic acid, hexanedioic acid, cyclohexanebutanoic acid, and (4-methylphenyl) cyclohexane acetic acid.

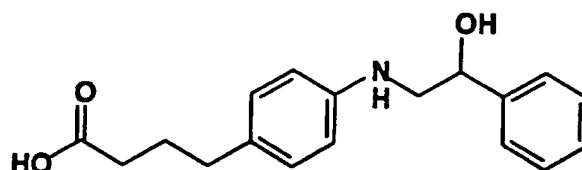
#### Other Examples of Complexing Perturbants

- 25 Although all complexing perturbants which can form the supramolecular complexes described herein are within the scope of the present invention, other examples of complexing perturbants include, but are
- 30 not limited to, 2-carboxymethyl-phenylalanine-leucine; 2-benzyl succinic acid, an actinonin, phenylsulfonyl aminophenyl-butyric acid,



and

5



LVIII

### Mimetics

Mimetics within the scope of the present invention are constructs which are structural and/or functional equivalents of an original entity. Structural and chemically functional mimetics of the supramolecular complexes and the reversible transportable intermediate states of active agents are not necessarily peptidic, as non-peptidic mimetics can be prepared which have the appropriate chemical and/or structural properties. However, preferred mimetics are peptides which have a different primary structure than the supramolecular complex or the intermediate state, but retain the same secondary and tertiary structure of the supramolecular complex or the intermediate state. Although mimetics may have less bioactivity than a native state or intermediate state active agent or supra molecular complex, the mimetics may possess other important properties which may not be possessed by the native state such as, for example, further increased ability to be delivered orally.

Methods of preparation of such mimetics are described, for example, in Yamazaki et al., Chirality 3:268-276 (1991); Wiley et al., Peptidomimetics Derived From Natural Products, Medicinal Research Reviews, Vol. 13, No. 3, 327-384 (1993); Gurrath et al., Eur. J. Biochem 210:991-921 (1992); Yamazaki et al., Int. J. Peptide Protein Res. 37:364-381 (1991); Bach et al., Int. J. Peptide Protein Res. 38:314-323 (1991); Clark et al., J. Med. Chem. 32:2026-2038 (1989); Portoghese, J. Med. Chem. 34:(6) 1715-1720 (1991); Zhou et al., J. Immunol. 149 (5) 1763-1769 (Sept 1, 1992); Holzman et al., J. Protein Chem. 10: (5) 553-563 (1991); Masler et al., Arch. Insect Biochem. and Physiol. 22:87-111 (1993); Saragovi et al., Biotechnology 10: (July 1992); Olmsteel et al., J. Med. Chem. 36:(1) 179-180 (1993); Malin et al. Peptides 14:47-51 (1993); and Kouns et al., Blood

80:(10) 2539-2537 (1992); Tanaka et al., Biophys. Chem. **50** (1994) 47-61;  
DeGrad et al., Science **243** (February 3, 1989); Regan et al., Science **241**:  
976-978 (August 19, 1988); Matouschek et al, Nature **340**: 122-126 (July  
13, 1989); Parker et al., Peptide Research **4**: (6) 347-354 (1991); Parker et  
5 al., Peptide Research **4**:(6) 355-363 (1991); Federov et al., J. Mol. Biol. **225**:  
927-931 (1992); Ptitsyn et al., Biopolymers **22**: 15-25 (1983); Ptitsyn et al.,  
Protein Engineering **2**:(6) 443-447 (1989).

For example, protein structures are determined by the collective  
intra- and inter-molecular interactions of the constituent amino acids. In  
10 alpha helices, the first and fourth amino acid in the helix interact non-  
covalently with one another. This pattern repeats through the entire helix  
except for the first four and last four amino acids. In addition, the side  
chains of amino acids can interact with one another. For example, the phenyl  
side chain of phenylalanine would probably not be solvent exposed if that  
15 phenylalanine were found in a helix. If the interactions of that phenylalanine  
contributed to helix stability then substituting an alanine for a phenylalanine  
would disrupt the helix and change the conformation of a protein.

Therefore, a mimetic could be created by first determining which  
amino acid side chains became solvent exposed and thus removed from  
20 contributing to stabilization of the native state such as by the technique of  
scanning mutagenesis. Mutants containing amino acid substitutions at those  
same sights could be created so that the substituted amino acids would  
render the protein conformation more intermediate-like that native-like.  
Confirmation that the appropriate structure had been synthesized could come  
25 from spectroscopy and other analytical methods.

#### Delivery Compositions

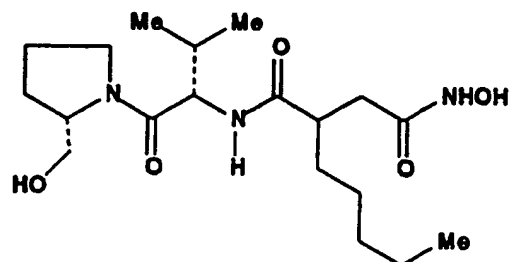
Delivery compositions which include the supramolecular complex  
described above are typically formulated by mixing the perturbant with the  
30 active agent. The components can be prepared well prior to administration or  
can be mixed just prior to administration.

The delivery compositions of the present invention may also  
include one or more enzyme inhibitors. Such enzyme inhibitors include, but

45

are not limited to, compounds such as actinonin or epiactinonin and derivatives thereof. These compounds have the formulas below:

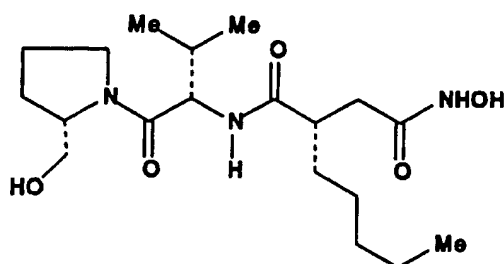
5



Actinonin

LIX

10



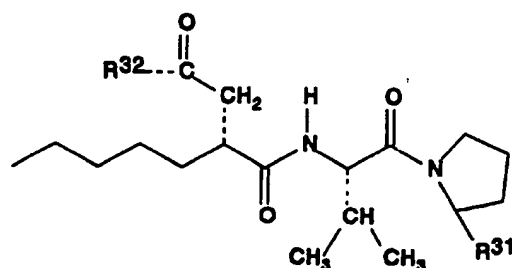
Epiactinonin

LX

15

Derivatives of these compounds are disclosed in U.S. Patent No. 5,206,384. Actinonin derivatives have the formula:

20



LXI

25

wherein  $R^{31}$  is sulfoxymethyl or carboxyl or a substituted carboxy group selected from carboxamide, hydroxaminocarbonyl and alkoxy carbonyl groups; and  $R^{32}$  is hydroxyl, alkoxy, hydroxyamino or sulfoxyamino group. Other enzyme inhibitors include, but are not limited to, aprotinin (Trasylol) and Bowman-Birk Inhibitor.

30

The delivery compositions of the present invention may be formulated into dosage units by the addition of one or more excipient(s),

diluent(s), disintegrant(s), lubricant(s), plasticizer(s), colorant(s), or dosing vehicle(s). Preferred dosage unit forms are oral dosage unit forms. Most preferred dosage unit forms include, but are not limited to, tablets, capsules, or liquids. The dosage unit forms can include biologically, pharmacologically, or therapeutically effective amounts of the active agent or can include less than such an amount if multiple dosage unit forms are to be used to administer a total dosage of the active agent. Dosage unit forms are prepared by methods conventional in the art.

The subject invention is useful for administering biologically active agents to any animals such as birds; mammals, such as primates and particularly humans; and insects. The system is particularly advantageous for delivering chemical or biologically active agents which would otherwise be destroyed or rendered less effective by conditions encountered before the active agent in the native state reaches its target zone (i.e. the area to which the active agent to be delivered) and by conditions within the body of the animal to which they are administered. Particularly, the present invention is useful in orally administering active agents, especially those which are not ordinarily orally deliverable.

## **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The following examples illustrate the invention without limitation. All parts and percentages are by weight unless otherwise indicated.

### **Example 1 - $\alpha$ -Interferon Native Gels**

Native gradient gels (Pharmacia) were run with 647  $\mu\text{g}/\text{ml}$  of  $\alpha$ -interferon, (Intron-A - Schering-Plough) and increasing amounts (10-500 mg/mL) of perturbant (mixture of L-Valine, L-Leucine, L-phenylalanine, L-lysine and L-arginine modified with benzenesulfonylchloride) (valine-7.4%, leucine-16.5%, phenylalanine - 40.3%, lysine - 16.2% and arginine - 19.6%). 4  $\mu\text{l}$  of material were loaded onto the gel using a 6/4 comb for loading.

Results are illustrated in Figure 1.



Lane 1 = High molecular weight marker (Bio-Rad) - 1:20 dilution w/dH<sub>2</sub>O - (5 $\mu$ l - > 100 $\mu$ l).

Lane 2 =  $\alpha$ -interferon A (647 $\mu$ g/mL) control 5 $\mu$ l + 5 $\mu$ l Bromophenol Blue (BPB) - (1.29 $\mu$ g loaded).

5 Lane 3 =  $\alpha$ -interferon + perturbant (10mg/mL) - 50 $\mu$ l  $\alpha$ -interferon + 50 $\mu$ l BPB = 100 $\mu$ l (1.29 $\mu$ g loaded).

Lane 4 =  $\alpha$ -interferon + perturbant (50mg/mL) 50 $\mu$ l  $\alpha$ -interferon + 50 $\mu$ l BPB = 100  $\mu$ l (1.29 $\mu$ g loaded).

10 Lane 5 =  $\alpha$ -interferon + perturbant (100mg/mL) 50 $\mu$ l  $\alpha$ -interferon + 50 $\mu$ l BPB = 100 $\mu$ l (1.29 $\mu$ g loaded).

Lane 6 =  $\alpha$ -interferon + perturbant (500 mg/mL) 5 $\mu$ l  $\alpha$ -interferon + 5 $\mu$ l BPB = 10 $\mu$ l (1.29 $\mu$ g loaded).

#### Example 1A - $\alpha$ -Interferon Native Gradient Gel

15 The method of Example 1 was followed substituting the thermal condensation product of glutamic acid, aspartic acid, tyrosine, and phenylalanine (Glu-Asp-Tyr-Phe) that was fractionated through a 3000 molecular weight cut-off filter for the perturbant.

Results are illustrated in Figure 2.

#### 20 Samples

Lane 1 = High Molecular Weight marker (Bio-Rad).

Lane 2 =  $\alpha$ -interferon (647 $\mu$ g/mL) - 5 $\mu$ l + 5 $\mu$ l BPB control.

Lane 3 =  $\alpha$ -interferon + perturbant (10mg/mL) - 50 $\mu$ l + 50 $\mu$ l BPB = 100 $\mu$ l.

Lane 4 =  $\alpha$ -interferon + perturbant - 50 $\mu$ l + 50 $\mu$ l BPB = 100 $\mu$ l.

25 Lane 5 =  $\alpha$ -interferon + perturbant (100mg/mL) - 50 $\mu$ l Intron A + 50 $\mu$ l BPB = 100 $\mu$ l.

Lane 6 =  $\alpha$ -interferon + perturbant (500mg/mL) - 5 $\mu$ l Intron A + 50 $\mu$ l BPB = 100 $\mu$ l.

30 Examples 1 and 1A illustrate that  $\alpha$ -interferon alone (lane 2 in Figures 1 and 2) banded at the appropriate molecular weight (approximately 19,000 Daltons). As the amount of perturbant added is increased in each subsequent lane relative to a fixed concentration of  $\alpha$ -interferon, the  $\alpha$ -interferon migrates

to a lower, rather than a higher, molecular weight. The change seen with the perturbant of Example 1 is more pronounced than that seen with the perturbant of Example 1A. This indicates that the  $\alpha$ -interferon structure is changing due to the two different perturbants, because if structure were not changing, there would be a shift towards higher molecular weight as perturbant complexes with the active agent.

**Example 2 - Oral Administration of  $\alpha$ -Interferon and Perturbant to Rats**

Male Sprague-Dawley rats (average weight approximately 250mg) were fasted overnight on wire racks with no bedding. Prior to dosing, animals were anesthetized with a combination ketamine/thorazine subcutaneous. Dosing solutions of the composition prepared according to Example 1 at 500  $\mu$ g/kg were administered via oral gavage through a 10-12 cm rubber catheter attached to a 1 cc syringe containing the dosing solution. Blood samples were drawn by tail vein bleeding at the designated time points. Serum was prepared and frozen at -70° C until ready for assay. Serum samples were assayed by ELISA (Biosource International, Camarillo, CA, Cytoscreen Immunoassay Kit™, Catalog #ASY-05 for human IFN- $\alpha$ ).

Results are illustrated in Figure 3.

**Example 2A - Oral Administration of  $\alpha$ -Interferon and Perturbant to Rats**

The method of Example 2 was followed substituting a dosing solution of the composition prepared according to Example 1A at 78  $\mu$ g/kg. Results are illustrated in Figure 3.

**Comparative Example 2\* - Oral Administration of  $\alpha$ -Interferon to Rats**

$\alpha$ -interferon at 100  $\mu$ g/kg without perturbant was administered according to the procedure of Example 2.

Results are illustrated in Figure 3.

**Example 3 - Oral Administration of Salmon Calcitonin and Perturbant to Rats**

The perturbant of Example 1 was reconstituted with distilled water and adjusted to a pH of 7.2-8.0 with HCl or NaOH. Salmon calcitonin (sCt) was dissolved in a citric acid stock solution (0.085 N and then combined with the perturbant solution to obtain the final dosing solution. Final concentrations of perturbant and sCt were 400mg/mL and 5µg/mL respectively.

Results are illustrated in Table 1 below.

24 hour fasted male Sprague Dawley rats weighing 100-150g were anesthetized with ketamine. Rats were administered the dosing solution in a vehicle by oral gavage at 800 mg/kg of perturbant and 10 µg/kg of sCt. The dosing solution was administered using a 10cm rubber catheter. One hour post-dosing, the rats were administered 1.5mg/kg thiorazine and 44mg/kg ketamine by intramuscular injection. At 1, 2, 3, and 4 hours post-dosing, blood samples were drawn from the rat tail artery for serum calcium concentration determination using the Sigma Diagnostic Kit (Catalog # 587-A, Sigma Chemical Co, St. Louis, MO). Results are illustrated in Figure 4.

**Example 3A - Oral Administration of Salmon Calcitonin and Perturbant to Rats**

The method of Example 3 was followed substituting L-tyrosine modified by cyclohexanoyl chloride as the perturbant.

Results are illustrated in Figure 4.

**Comparative Example 3\***

Salmon calcitonin (10 µg/kg) without perturbant was administered to rats according to the procedure of Example 3.

Results are illustrated in Figure 4.

**Example 4 - Isothermal Titration Calorimetry**

A dosing composition of the perturbant of Example 1 at 2.4mM and sCt at 0.3mM was prepared, and isothermal titration calorimetry was performed at

pH 6.5 and 4.5. The buffer at pH 6.5 was 30mM Hepes-30mM NaCl, and the buffer at pH 4.5, was 30mM sodium acetate-30mM NaCl.

All experiments were performed at 30°C using 8.0mM perturbant in the dropping syringe and 1.0mM calcitonin in the calorimeter cell. In all  
5 experiments, 15x10 $\mu$ l increments of perturbant were added in 10 second duration additions with 2 minutes equilibration between additions.

Results were validated in experiments where perturbant (8mM) was placed in the dropping syringe, and equivalent increments were added to pH 4.5 buffer (no sCt) and where perturbant was placed in the dropping syringe and  
10 10 $\mu$ l increments were added to pH 6.5 buffer (no sCt). Titration curves were not obtained in these experiments, and the results showed that heat of mixing and/or dilution of perturbant is negligible. Therefore, the experimental isotherms were not corrected by background subtraction.

Results are illustrated in Table 1 below.

15

#### Example 4A

The method of Example 4 was followed substituting the perturbant of Example 1A. Results were validated in experiments where perturbant was placed in the dropping syringe, and equivalent increments were added to pH 4.5  
20 buffer (no sCt).

Results are illustrated in Table 1 below.

TABLE - 1				
Binding Parameters of Perturbants as Determined by ITC <sup>1</sup>				
	K <sub>D</sub> (M)	ΔH (cal/mol)	ΔS (cal/mol °K)	N
<b>pH 6.6</b>				
Example 4	4.59 x 10 <sup>-8</sup>	+240	+34.4	0.6
Example 4A	6.99 x 10 <sup>-9</sup>	+277	+38.3	11.6
<b>pH 4.5</b>				
Example 4	Precipitates			
Example 4A	1.29 x 10 <sup>-4</sup>	+553	+19.8	+0.8

#### **Example 5 - GuHCl Denaturation Of $\alpha$ -Interferon**

A stock solution of 9.1 mg/mL of  $\alpha$ -interferon (Schering Plough Corp.) in 20mM sodium phosphate buffer at pH 7.2 was prepared. Samples were prepared by diluting the  $\alpha$ -interferon with the sodium phosphate buffer and 10 M guanidine hydrochloride (GuHCl) (Sigma Chemical Co. - St. Louis, MO) stock solution to 200ug/mL concentration of  $\alpha$ -interferon at various concentrations of GuHCl. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence measurements were made at 25°C using a Hitachi F-4500. Protein tryptophan fluorescence was observed at an excitation wavelength of 298nm and an emission wavelength of 343nm. ANS (1-anilinonapthalene-8-sulfonate) fluorescence was observed at an excitation wavelength of 355nm and an emission wavelength of 530nm. For all

<sup>1</sup> Calorimetry experiments were performed essentially as detailed by You, J.L., Scarsdale, J.N., and Harris, R.B., J. Prot. Chem. 10: 301-311, 1991; You, Junling, Page, Jimmy D., Scarsdale, J. Neel, Colman, Robert W., and Harris, R.B., Peptides 14: 867-876, 1993; Tyler-Cross, R., Sobel, M., Siler, D.F., and Harris, R.B., Arch. Biochem. Biophys. 306: 528-533, 1993; Tyler-Cross, R., Sobel, M., Marques, D., and Harris, R.B., Protein Science 3: 620-627, 1994.

fluorescence measurements, a 5nm spectral bandpass was chosen for both excitation and emission.

Results are illustrated in Figure 5.

5 **Example 6 - Concentration Effect of GuHCl on  $\alpha$ -Interferon Configuration**

GuHCl 5M stock solution was prepared using 20 mM sodium phosphate, pH 7.2 buffer. After dilution, the pH of the stock was checked and adjusted by concentrated HCl. To determine the concentration of final solution the refractive index referenced in Methods in Enzymology, Vol. 6, page 43 by

10 Yasuhiko Nozaki was used.

$\alpha$ -interferon stock (9.1 mg/mL) was mixed with sufficient amounts of GuHCl to yield the concentrations of Table 1A below:

**Table 1A -  $\alpha$ -Interferon/GuHCl Solutions**

15

GuHCl (M)	$\alpha$ -IFN (mg/mL)
0.5	0.60
1.0	0.53
1.5	0.60
2.0	0.50
3.0	0.60
4.0	0.50

20

25 Differential scanning calorimetry (DSC) was run, and results are illustrated in Figure 6.

**Example 7 - pH Titration of Intron A as Measured by Intrinsic Tryptophan Fluorescence**

30

A stock solution of 9.1 mg/mL  $\alpha$ -interferon in 20mM sodium phosphate buffer at pH 7.2 (Schering Plough Corp.) was prepared. Samples were prepared by diluting the  $\alpha$ -interferon to a concentration of 200 ug/mL into solution buffered at various pH values using the following buffers: Glycine at

pH 2 and 12, sodium phosphate at pH 3, 4, 5, 7, and boric acid at pH 8. These buffers were prepared as described in the Practical Handbook of Biochemistry and Molecular Biology, Edited by Gerald D. Fasman, 1990. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence was measured according to the procedure of Example 5. Results are illustrated in Figure 7.

**Example 8 - pH Titration of Insulin Measured by ANS Fluorescence**

A stock solution was prepared by dissolving 2mg of insulin in 1mL of deionized water. 1-anilinonaphthalene-8-sulphonate (ANS) stock solution was prepared by dissolving 10mg in 10mL of deionized water. Samples were prepared by diluting the insulin to a concentration of 200 ug/mL into solution buffered at various pH values using the following buffers: Glycine at pH 2 and 12, sodium phosphate at pH 3, 4, 5, 7, and boric acid at pH 8. These buffers were prepared as described in the Practical Handbook of Biochemistry and Molecular Biology, Edited by Gerald D. Fasman, 1990. The final ANS concentration was 90ug/mL. Diluted samples were allowed to come to equilibrium by incubation for approx. 30 minutes at room temperature prior to measurement.

Fluorescence was measured according to the procedure of Example 5. Results are illustrated in Figure 8.

**Example 9 - Reversibility of Circular Dichroism Spectra of  $\alpha$ -Interferon at pH 2 and 7.2**

Circular dichroism spectra of  $\alpha$ -interferon were generated at pH 7.2. The pH of the solution was then readjusted to pH 2, and the sample was rescanned. The sample solution was then readjusted to 7.2 and rescanned.

Concentration of  $\alpha$ -interferon was 9.2 $\mu$ M or 0.17848mg/mL, ([IFN] stock = 9.1mg/mL). Buffers used were 20mM NaPhosphate at pH 7.2; and 20mM Glycine at pH 2.0.

Reversal of the pH to 7.2 resulted in complete restoration of the native structure, demonstrating the reversibility of the intermediate state. It is believed that the free energy difference between the native state and the intermediate state is small.

5 Results are illustrated in Figures 9A and 9B.

**Example 10 - Circular Dichroism Spectra of  $\alpha$ -Interferon at 7.2 - pH Dependence**

10 The extent of ordered secondary structure of  $\alpha$ -interferon at different pH's was determined by circular dichroism (CD) measurements in the far UV range. The large dilution factor of interferon stock (~50 times) resulted in the sample being at the proper pH. Concentration of  $\alpha$ -interferon was 9.2  $\mu$ M or 0.17848mg/mL, ([IFN] stock = 9.1mg/mL). Buffers used were 20mM sodium  
15 phosphate at pH 6.0 and 7.2; 20mM NaAc at pH 3.0, 4.0, 4.5, 5.0 and 5.5; and 20mM Glycine at pH 2.0

The secondary structure content was estimated with several fitting programs, each of which decomposes the CD curve into four major structural components:  $\alpha$ -helix,  $\beta$ -sheet, turns, and random coil. Two of those programs  
20 were provided with the CD instrument as an analysis facility. The first program uses seven reference proteins: Myoglobin, Lysozyme, Papain, Cytochrome C, Hemoglobin, Ribonuclease A and Chymotrypsin. The second uses Yang.REF reference file.

A third program, CCAFAST, uses the Convex Constraint Algorithm  
25 and is described in "Analysis of Circular Dichroism Spectrum of Proteins Using the Convex Constraint Algorithm: A Practical Guide". (A. Perczel, K. Park and G.D. Fasman (1992) Anal. Biochem. 203: 83-93).

Deconvolution of the far UV scans over a range of pH volumes (2.0-7.2) indicates significant compaction of the secondary structure at pH 3.5.  
30 The near UV scan indicates a disruption of tertiary structure packing, and the far UV scan indicates that there is still significant secondary structure at this pH.

Results are illustrated in Figure 10.



**Example 11 - DSC of Insulin and Increasing Concentrations of GuHCl**

DSC was performed with 6mg/mL Insulin (0.83mM assuming a  
5 molecular weight of 6,000) in 50mM phosphate buffer, pH 7.5. Each subsequent thermogram was corrected by background subtraction of a 0.6M guanidine-phosphate buffer solution.

Insulin was freshly prepared as a concentrated stock solution in  
50mM phosphate buffer, pH 7.5, and an appropriate aliquot was diluted in  
10 buffer, filtered through a 2 micron PTFE filter, and degassed for at least 20 minutes. The reference cell contained degassed buffer.

Scanning calorimetry was performed using 5mg 0.83mM porcine insulin  
(MW 6,000) per mL in 50mM phosphate buffer, pH 7.5. All thermograms were  
performed on a Microcal MC-2 scanning calorimeter equipped with the DA2 data  
15 acquisition system operated in the upscale mode at 1° C/min ( up to 90° C),  
and data points were collected at 20 second intervals. All scans were initiated  
at least 20 degrees below the observed transitions for the active agent. All  
thermograms were corrected for baseline subtraction and normalized for the  
concentration of macromolecule. According to the methods of the Johns  
20 Hopkins Biocalorimetry Center, See, for example, Ramsay et al. *Biochemistry*  
(1990) 29:8677-8693; Schon et al. *Biochemistry* (1989) 28:5019-5024 (1990)  
29: 781-788. The DSC data analysis software is based on the statistical  
mechanical deconvolution of a thermally induced macromolecular melting profile.

The effect of GuHCl on structure was assessed in DSC experiments  
25 where individual solutions were prepared in phosphate buffer, pH 7.5, containing  
denaturant diluted from a 5M stock solution to concentrations ranging for 0.5-  
2M.

Results are illustrated in Table 2 below.

Table - 2	
DSC of Insulin and Increasing Concentrations of Guanidine Hydrochloride	
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)
Insulin 0.0M GuHCl	78.3
Insulin + 0.5M GuHCl	79.3
Insulin + 1.0M GuHCl	77.5
Insulin + 2.0M GuHCl	69.7
Insulin + 3.0M GuHCl	no transition observed

**Example 12 - Effect of Ionic Strength on the DSC Spectrum of Insulin**

A sample containing 6mg/mL insulin (0.83mM in 50mM phosphate buffer, pH 7.5, containing 0.25, 0.5, or 1.0M NaCl) was used. Thermograms were performed according to the procedure in Example 11 and were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank as described above.

The effect of increasing ionic strength on structure was assessed in DSC experiments where individual solutions were prepared so as to contain NaCl at concentrations ranging from 0.25-3M.

Results are illustrated in Table 3 below.

Table - 3	
Effect of Ionic Strength on the DSC Spectrum of Insulin	
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)
Insulin 0.0M NaCl	78.3
Insulin + 0.25M NaCl	80.7
Insulin + 0.5M NaCl	80.7
Insulin + 1.0M NaCl	80.7

**Example 12A - Effect of Ionic Strength on the DSC Spectrum of rhGh**

The method of Example 11 was followed substituting 5mg/mL recombinant human growth hormone (rhGh) (225  $\mu$ M based on M,22,128 of HGH) in 50mM phosphate buffer, pH 7.5 containing either 0.5 or 1.0M NaCl, for the insulin. The thermograms were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank.

Results are illustrated in Table 4 below.

10

Table - 4		
Effect of Ionic Strength on the DSC Spectrum of rhGh		
	<u>T<sub>m</sub> (C<sub>p</sub> max)</u> (°C)	<u><math>\Delta H^\circ</math></u> (kcal/mol)
rhGh 0.0M NaCl	75.2	191.0
rhGh + 0.5M NaCl	75.8	89.7
rhGh + 10.0M NaCl	76.5	50.5

15

**Example 13 - Effect of pH on the DSC Spectrum of rhGH**

5mg/mL rhGh were dissolved in buffer (0.17mM in 50mM phosphate buffer, assuming a molecular weight of 20,000). The pH of the solution was adjusted to the desired value, and all curves were corrected by baseline subtraction.

The effect of pH on structure was assessed by DSC according to the procedure of Example 11 where individual solutions were prepared in phosphate buffer ranging in pH value from 2.0 to 6.0.

Results are illustrated in Table 5 below.

Table - 5		
Effect of pH on the DSC Spectrum of rhGh		
	<u>T<sub>m</sub> (C<sub>p</sub>, max)</u> (°C)	<u>ΔH°</u> (kcal/mol)
5	pH 2.0	no transition observed
	pH 3.0	no transition observed
	pH 3.5	no transition observed
	pH 4.0	≈ 73.0
	pH 5.0	75.0
10	pH 6.0	75.2
	pH 7.5 (10 mg/mL)	a) 73 b) 75
		(a) + (b) = 632

**Example 14 - Effect of GuHCl on the DSC Spectrum of rhGh**

An initial scan of rhGh was performed at 10mg/mL in the absence of GuHCl (0.33mM assuming 20,000 molecular weight). Subsequently, the concentration of rhGh was lowered to 5mg/mL (0.17mM) in 50mM phosphate buffer, pH 7.5 containing varying concentrations of GuHCl. Each subsequent thermogram was corrected by background subtraction of a 0.5M guanidine-phosphate buffer solution. The thermograms were corrected by subtraction of a 0.5M NaCl-phosphate buffer blank. Scans were performed according to the procedure of Example 11.

Results are illustrated in Table 6 below.

Table - 6			
Effect of Guanidine Hydrochloride on DSC Spectrum of rhGh			
	DOMAIN A $T_m$ (Cp.max) (°C)	DOMAIN B $T_m$ (Cp.max) (°C)	$\Delta H^\circ$ (kcal/mol)
rhGh	72.6	74.3	632
rhGh + 0.5M GuHCl	71.5	not defined, but present	48
rhGh + 1.0M GuHCl	70.9	absent	109
rhGh + 1.5M GuHCl	69.7	absent	12
rhGh + 2.0M GuHCl	70.0	absent	58
rhGh + 2.5M GuHCl	70.7	absent	99

**Example 15 - pH Dependence of  $\alpha$ -Interferon Conformation**

$\alpha$ -interferon stock (9.1 mg/mL) was diluted with buffer to a concentration of 0.6 mg/mL. The sample was dialyzed overnight in buffer (volume ratio of  $\alpha$ -interferon to buffer was 1:4000). Since there was no extinction coefficient provided, concentration of the sample used was determined by comparison of absorption spectra of the sample before and after dialysis. For each particular pH, the absorbance of the nondialyzed  $\alpha$ -interferon of known concentration was measured at 280nm. Then after dialysis, absorbance was read again to account for the protein loss, dilution, etc. Buffer conditions and  $\alpha$ -interferon concentrations were:

- pH 3.0: Buffer - 20mM NaAc. [IFN] = 0.50 mg/mL;
- pH 4.1: Buffer - 20mM NaAc. [IFN] = 0.53 mg/mL;
- pH 5.0: Buffer - 20mM NaAc. [IFN] = 0.37 mg/mL;
- pH 6.0: Buffer - 20mM Na Phosphate. [IFN] = 0.37 mg/mL;
- pH 7.2: Buffer - 20mM Na Phosphate. [IFN] = 0.48 mg/mL.

DSC scans were performed according to the procedure of Example 11. Although clear, transparent solutions of  $\alpha$ -interferon were obtained for every pH at room temperature, there were noticeable signs of precipitation at pH 5.0 and 6.0 after the temperature scans.

Results are illustrated in Table 7 below.

TABLE - 7		
$\alpha$ -Interferon - pH dependence DSC		
pH	T <sub>m</sub> °C	$\Delta H$ cal/mol
7.2	66.84	732717
6.0	65.34	45580
5.0	67.32	69782
4.1	65.64	60470
3.0	--	--

10

**Example 16 - Concentration Effect of GuHCl on  $\alpha$ -Interferon Conformation**

GuHCl/ $\alpha$ -interferon samples were prepared according to the method of Example 6. DSC scans were performed according to the procedure of Example 11.

15

Results are illustrated in Table 8 below.

TABLE - 8		
$\alpha$ -Interferon in GuHCl DSC		
[GuHCl] M	T <sub>m</sub> °C	$\Delta H$ cal/mol
0.0	67.12	72562
0.5	64.43	50827
1.0	63.04	41705
1.5	60.11	29520
2.0	56.32	24980
3.0	45.90	20577
4.0	--	--

20

25

Examples 5-16 illustrate that ionic strength, guanidine hydrochloride concentration, and pH result in changes in the T<sub>m</sub> of active agents, indicating a change in conformation. This was confirmed by fluorescence spectroscopy. The reversible intermediate conformational states can be used as templates to prepare mimetics.

30

**Example 17 - Preparation of  $\alpha$ -Interferon Intermediate State Mimetics**

An intermediate conformational state of  $\alpha$ -interferon is determined. A peptide mimetic having the secondary and tertiary structure of the intermediate state is prepared.

5

**Example 18 - Preparation of Insulin Intermediate State Mimetics**

The method of Example 17 is followed substituting an insulin for the  $\alpha$ -interferon.

10

**Example 19 - Preparation of rhGh Intermediate State Mimetics**

The method of Example 17 is followed substituting recombinant human growth hormone for the  $\alpha$ -interferon.

15

**Example 20 - In vivo Administration of  $\alpha$ -Interferon Mimetics**

Rats are dosed according to the procedure of Example 2 with the mimetic prepared according to the procedure of Example 17.

**Example 21 - In vivo Administration of Insulin Mimetics**

The procedure of Example 20 was followed, substituting the mimetic prepared according to the procedure of Example 18.

25

**Example 22 - In vivo Administration of rhGH Mimetics**

The procedure of example 19 was followed, substituting the mimetic prepared according to the procedure of Example 19.

**Example 24 - Titration of  $\alpha$ -Interferon as Measured by Intrinsic Tryptophan Fluorescence**

A stock solution of 9.1mg/mL  $\alpha$ -interferon in 20mM sodium phosphate buffer at pH 7.2 was prepared. A stock solution of perturbant was prepared by

30

dissolving 800mg of perturbant (L-arginine acylated with cyclohexanoyl chloride) in 2mL of 20mM Sodium Phosphate buffer (pH7).

Samples were prepared by diluting the  $\alpha$ -interferon with the sodium phosphate buffer and perturbant stock solution at various perturbant concentrations. Diluted samples were allowed to come to equilibrium by incubation for approximately 30 minutes at room temperature prior to measurement.

Fluorescence from the endogenous tryptophan resident of  $\alpha$ -interferon were measured according to the procedure of Example 5. The perturbant did not contain a fluorophore.

Results are illustrated in Figure 11.

**Example 25 - In vivo Administration of Perturbant and  $\alpha$  Interferon to Rats**

Rats were dosed according to the method of Example 2 with dosing solutions containing the perturbant of Example 24 (800 mg/kg) mixed with  $\alpha$ -interferon (1 mg/kg). Serum samples were collected and assayed by ELISA according to the procedure of Example 2.

Results are illustrated in Figure 12.

**20 Comparative Example 25\***

Rats were dosed according to the method of Example 25 with  $\alpha$ -interferon (1mg/kg). Serum samples were collected and assayed according to the procedures of Example 25.

Results are illustrated in Figure 12.

25

**Example 26 - Differential Scanning Colorimetry of  $\alpha$ -Interferon and Perturbant**

Perturbant binding DSC was conducted using 20mM NaPhosphate buffer at pH 7.2. Dry perturbant was weighed out to make perturbant stock solutions.  $\alpha$ -interferon stock was diluted in the buffer.  $\alpha$ -interferon solution was not dialyzed prior to experiments for the purpose of having the same active concentration for the whole set.



DSC thermograms were generated with  $\alpha$ -interferon at a concentration of 0.64 mg/ml and a perturbant (phenylsulfonyl-para-aminobenzoic acid purified to >98% (as determined by reverse phase chromatography prior to generation of the spectra)) at perturbant concentrations of 5, 10, 25 and 100 mg/ml. DSC was conducted on a DASM-4 differential scanning calorimeter interfaced to an IBM PC for automatic collection of the data. The scanning rate was 60 °C/h. Results are illustrated in Table 9 below and Example 13.

**Comparative Example 26\* - Different Scanning Calorimetry of  $\alpha$ -Interferon**

The method of Example 26 was followed substituting  $\alpha$ -interferon without perturbant. Results are illustrated in Table 9 below and Example 13.

TABLE - 9		
$\alpha$ -Interferon + Perturbant DSC		
Perturbant - mg/ml	T <sub>m</sub> °C	$\Delta H$ cal/mol
0	67.12	72562
5	64.37	60151
10	62.3	53161
25	58.15	35393
100	46.18	5439.3

DSC scans where the added concentration of perturbant ranged from 0-100 mg/mL show induced conformational changes in the  $\alpha$ -interferon that occur in a concentration dependent manner. At 100 mg/mL of the perturbant, the thermogram indicated that the  $\alpha$ -interferon Cp vs. T<sub>m</sub> curve was a flat line. The flat Cp vs. T<sub>m</sub> curve obtained at 100 mg/mL of perturbant indicates that hydrophobic residues within the  $\alpha$ -interferon molecule became solvent exposed. It is clear that the perturbant was able to change the structure of  $\alpha$ -interferon in a concentration dependent manner.

**Example 27 -      Dialysis Experiments - Reversibility of  
Complexing with the Perturbant**

An  $\alpha$ -interferon stock solution at a concentration of 9.1 mg/mL was diluted with buffer to an  $\alpha$ -interferon concentration of 0.6 mg/mL. DSC was performed according to the procedure of Example 26.

Results are illustrated in Figure 14A.

$\alpha$ -interferon (0.6 mg/ml) and the perturbant of Example 26 (100 mg/ml) were mixed with no apparent changes in the Cp of the solution. This solution was then dialyzed overnight into phosphate buffer, and the thermogram was rerun. Results are illustrated in Figure 14B.

The dialyzed sample had essentially the same Tm and the same area under the Cp vs. Tm curve as it did prior to addition of the perturbant. This indicated that not only was the perturbant able to induce conformational changes in the protein but that this process was reversible. Dilution was enough of a driving force to effect disengagement of the perturbant from the active agent.

**Example 28 -      Perturbant and  $\alpha$ -Interferon DSC**

The method of Example 6 was followed, substituting the perturbant of Example 26 for the GuHCl.

Results are illustrated in Figure 15.

The DSC experiments on the equilibrium denaturation of  $\alpha$ -interferon indicate the existence of intermediate conformations of the molecule.  $\Delta H$  vs. Tm plots indicate the energetics of intermediate conformations occupied by  $\alpha$ -interferon at each set of experimental conditions.

**Example 29 -      In Vivo Administration of Perturbant and  $\alpha$  Interferon to Rats**

Rats were dosed according to the method of Example 2 with a dosing solution of the perturbant of Example 4 (800 mg/kg) and  $\alpha$ -interferon (1 mg/kg). Serum samples were collected and assayed by ELISA according to the procedure of Example 2.

Results are illustrated in Figure 16.

**Comparative Example 29 - *In Vivo* Administration of  $\alpha$  Interferon to Rats**

Rats were dosed according to the method of Example 29 with  $\alpha$ -interferon (1 mg/kg) without perturbant. Serum samples were collected and assayed according to the procedure of Example 29.

5 Results are illustrated in Figure 16.

Figure 16 illustrates that when active agent mixed with perturbant was orally gavaged into animals, significant serum titers of  $\alpha$ -interferon were detectable in the systemic circulation, and the  $\alpha$ -interferon was fully active. Confirming data that the delivered  $\alpha$ -interferon was fully active included the fact  
10 that the serum was assayed by a commercial ELISA kit which utilizes a monoclonal antibody able to recognize an epitope specific to the native conformation of Intron and that the serum was further assayed using the cytopathic effect assay which determined titers of Intron that correlated with the titers measured by ELISA (data not shown). Therefore, the conformational  
15 changes which occurred as a result of with the perturbant, were reversible changes.

**Example 30 - Perturbant Concentration Dependent Change in  $\alpha$ -Interferon**

The method of Example 26 was followed substituting cyclohexanoyl  
20 phenylglycine for the perturbant.

Results are illustrated in Table 10 below and in Figure 17.

TABLE - 10		
$\alpha$ -Interferon + Perturbant DSC		
Perturbant mg/ml	T <sub>m</sub> °C	$\Delta H$ cal/mol
0	67.12	72562
5	63.00	42299
10	59.49	43058
25	52.79	27237
100	--	0

30 Cyclohexanoyl phenylglycine induced conformational changes in  $\alpha$ -interferon that were concentration dependent.

**Example 31 - In Vivo Administration of Perturbant and  $\alpha$ -Interferon to Rats**

Rats were dosed according to the method of Example 2 with dosing solutions containing the perturbant of Example 30 (800 mg/kg) and  $\alpha$ -interferon 1 (mg/kg). Serum samples were collected and assayed by ELISA according to the procedure of Example 2.

Results are illustrated in Figure 18.

**Comparative Example 31\* - In vivo Administration of Perturbant and  $\alpha$ -Interferon to Rats**

Rats were dosed according to the method of Example 2 with  $\alpha$ -interferon (1 mg/kg) without perturbant. Serum samples were collected and assayed by ELISA according to the procedure of Example 2.

Results are illustrated in Figure 18.

**Example 32 - Perturbant and  $\alpha$ -Interferon DSC**

The method of Example 6 was followed substituting the perturbant of Example 30 for the GuHCl.

Results are illustrated in Figure 19.

The  $\Delta H$  v.  $T_m$  plot indicates the existence of an equilibrium intermediate conformation of  $\alpha$ -interferon that is stable at below 5 and 25 mg/ml of added cyclohexanoyl phenylglycine perturbant.

**Example 33 - Isothermal Titration Calorimetry of  $\alpha$ -Interferon with Perturbant**

Isothermal titration calorimetry of perturbant complexing with  $\alpha$ -interferon was performed at 25°C at two different pH's. The buffers used were 20mM NaPhosphate for pH 7.2 and 20mM NaAc for pH 3.0.  $\alpha$ -interferon solution was dialyzed before the experiment to reach the appropriate pH. Dry perturbants were weighed and diluted in dialysate.

ITC was conducted on a MicroCal OMEGA titration calorimeter (MicroCal Inc. - Northampton, MA). Data points were collected every 2 seconds, without subsequent filtering.  $\alpha$ -interferon solution placed in 1.3625mL cell was titrated

using a 250 $\mu$ L syringe filled with concentrated perturbant solution. A certain amount of titrant was injected every 3-5 minutes for up to 55 injections.

A reference experiment to correct for the heat of mixing of two solutions, was performed identically except that the reaction cell was filled with buffer without active agent.

Analysis of the data was performed using the software developed at the Johns Hopkins University Biocalorimetry Center.

The titration at pH 7.2 included 53 injections of 2 $\mu$ L of the perturbant of Example 30 (50mg/mL = 191.6mM (MW 261)) and  $\alpha$ -interferon (1.3 mg/mL = 0.067mM (MW 19400)).

Results are illustrated in Figure 20.

Curve fitting indicated multiple independent sites:

$n(1) = 121.0354$  where  $n = \#$  of completed perturbant molecules

$\Delta H(1) = 58.5932$  cal/Mole perturbant

$\log_{10} K_a(1) = 2.524834$  where  $K_a =$  association constant

x-axis units are concentration of carrier in mM.

y-axis units represent heat/injection expressed in calories.

At pH 3, complexing resulted in a negative enthalpy.

## 20 Comparative Example 33\* - Isothermal Titration Calorimetry of Perturbant

The method of Example 33 was followed, substituting 53 injections of 2  $\mu$ l for the perturbant (50mg/mL = 191.6mM) [IFN] = 0 mg of Example 30 without active agent.

25 Example 33 and Comparative Example 33 illustrate that  $\alpha$ -interferon has a positive enthalpy and a binding constant ( $K_d \approx 10^{-3}$ M).

## 30 Example 34 - Isothermal Titration Calorimetry of $\alpha$ -Interferon and Perturbant Complexing

The method of Example 33 was followed substituting the perturbant of Example 26 for the perturbant of Example 30.

The titration at pH 7.2 included two runs of 55 injections each of 5  $\mu$ L of perturbant (50mg/mL = 181mM (FW 277)) and  $\alpha$ -interferon (2.31mg/mL = 0.119mM, (MW 19400)).

Results are illustrated in Figure 21.

5 Curve fitting indicated multiple independent sites:

$n(1) = 55.11848$  where  $n$  = # of complex perturbant molecules

$\Delta H(1) = -114.587$  cal/Mole perturbant

$\log_{10} K_a(1) = 2.819748$  where  $K_a$  = association constant

x-axis units are concentration of carrier in mM.

10 y-axis units represent heat/injection expressed in calories.

Complexing of perturbant to  $\alpha$ -interferon at pH 3.0 resulted in precipitation of the complex out of the solution. Due to the heat effect produced by this process, it was impossible to measure the complexing parameters.

15

**Comparative Example 34\* - Isothermal Titration Calorimetry of Perturbant**

The method of Example 34 was followed, substituting 55 injections of 5  $\mu$ L of the perturbant of Example 26(50mg/mL = 181mM) in 20 mM sodium phosphate pH 7.2 without active agent.

20

The perturbant of Example 26 complexed with  $\alpha$ -interferon resulted in a negative enthalpy and a comparable binding constant to that of the perturbant of Example 30 and  $\alpha$ -interferon.

25 Examples 33 and 34 indicate that the stronger the perturbant complexes with the active agent and the more thermodynamically stable the intermediate state of the active agent, the greater the bioavailability of the active agent.

Therefore, by plotting the  $\Delta H$  v.  $T_m$  curve for an active agent and a perturbant, those perturbants that induce little or no enthalpic change over the broadest range of  $T_m$  would be preferred perturbants. It is believed that  
30 perturbants that stabilize the intermediate state to a greater extent will result in more efficient delivery of the active agent.

**Example 35 - Comparison of the Effects of Three Perturbants On  $\Delta H$  vs.  $T_m$  Plots with  $\alpha$ -Interferon**

DSC experiments were carried out according to the procedure of Example 5 26, with 0.5 mg/ml  $\alpha$ -interferon mixed with (1) benzoyl para-amino phenylbutyric acid, (2) the perturbant of Example 30, or (3) the perturbant of Example 26.

Benzoyl para-amino phenylbutyric acid was poorly soluble under the buffer conditions. Maximum concentration at which the solution was still transparent 10 at room temperature was  $\sim 8\text{mg/mL}$ . Therefore, the concentrations of the perturbant used were 2, 4, and  $6\text{mg/mL}$ . Results are illustrate in Figures 22 and 23. The dashed line in Figure 22 represents the linear least squares, and the regression equation is at the top of Figure 22.

15 
$$Y = -1.424e^5 + 3148.8x$$
$$R = 0.9912$$

Figures 22 and 23 illustrate that conformational changes in  $\alpha$ -interferon are more readily produced by benzoyl para-amino phenylbutyric acid than by the 20 perturbants of Examples 30 and 26, and that such changes are more readily produced by the perturbant of Example 30 than by the perturbant of Example 26.

25 **Example 36 - Isothermal Titration Calorimetry of  $\alpha$ -Interferon and Complexing**

ITC was performed according to the method of Example 33 with 40 injections of  $5\text{ }\mu\text{L}$  of the perturbant benzoyl para-amino phenylbutyric acid ( $7.5\text{mg/mL} = 24.59\text{mM}$ , (FW 305)) and  $\alpha$ -interferon ( $2.5\text{mg/mL} = 0.129\text{mM}$ , 30 (MW 19400)).

Results are illustrated in Figure 24.

Curve fitting indicated multiple independent sites:

$n(1) = 23.69578$  where  $n = \#$  of complexed perturbant molecules

$\Delta H(1) = 791.5726\text{ cal/Mole perturbant}$

$\log_{10} K_a(1) = 3.343261$  where  $K_a$  = association constant

x-axis represents concentration of carrier in mM.

y-axis represents heat/injection expressed in calories.

#### 5 Comparative Example 36\*

ITC was performed according to the method of Example 36 with 40 injections of 5  $\mu\text{l}$  of the perturbant benzoyl para-amino phenylbutyric acid (7.5mg/mL = 24.59mM) in 20mM NaPhosphate pH 7.2 buffer, without active agent.

10 The apparent dissociation constant for the perturbant of Example 35 is greater than that for the perturbant of Example 30 ( $10^{-4}\text{M}$ ) at pH 7.

Therefore, benzoyl para-amino phenylbutyric acid complexes more strongly to  $\alpha$ -interferon and induces the native state-reversible intermediate conformational state at lower concentrations of perturbant.

15

#### Examples 37-39 - Comparative *In Vivo* Pharmacokinetics of Various Perturbants and $\alpha$ -Interferon

20 Rats were dosed according to the procedure of Example 2 with dosing solutions containing the perturbant of Example 26 (800 mg/kg) (1), the perturbant of Example 30 (800 mg/kg) (2), or the perturbant of benzoyl para-amino phenylbutyric acid (300 mg/kg) (3) and  $\alpha$ -interferon at 1mg/kg. Serum samples were collected and assayed by ELISA according to the procedure of Example 2.

25 Results are illustrated in Figure 25.

#### Comparative Example 37\* - *In Vivo* Pharmacokinetics of $\alpha$ -Interferon

Rats were dosed according to the method of Example 37 with  $\alpha$ -interferon without perturbant. Serum samples were collected and assayed by ELISA  
30 according to the procedure of Example 2.

Results are illustrated in Figure 25.

Examples 35-39 illustrate that *in vivo* potency was correctly predicted by *in vitro* modeling.



**Examples 40-42 - Comparative *In Vivo* Pharmacokinetics of Various Perturbants with rhGh In Hypophysectomized Rats**

Rats were dosed according to the procedure of Example 2, with dosing solutions containing the perturbants salicyloyl chloride modified L-phenylalanine  
5 (1.2 g/kg) (40), phenylsulfonyl para-amino benzoic acid (1.2 g/kg) (41), or cyclohexanoyl chloride modified L-tyrosine (1.2 g/kg) (42) mixed with rhGh (1 mg/kg).

Rats were hypophysectomized according to the procedure of Loughna, P.T. et al, *Biochem. Biophys. Res. Comm.*, Jan. 14, 1994, 198(1), 97-102.  
10 Serum samples were assayed by ELISA (Medix Biotech, Inc., Foster City, CA, HGH Enzyme Immunoassay Kit).

Results are illustrated in Figure 26.

**Examples 43-45 - Isothermal Titration Calorimetry of rhGH at pH 7.5 and 4.0 with Different Perturbants**

The ability of rhGh to complex with various perturbants was assessed by ITC using a Microcal Omega titrator, usually equilibrated at 30°C. The sample  
20 cell of the calorimeter was filled with degassed rhGH (usually at 0.25mM) prepared in 50mM phosphate buffer, pH 7.5 or 4.0. The perturbant (cyclohexanoylchloride modified L-tyrosin (a), salicyloyl modified L-phenylalanine (b), or phenylsulfonyl-para-amino benzoic acid (c)) was then placed in the dropping syringe at 1mM (for pH 7.5) and 2.5mM, (for pH 4.0). Twenty to  
25 twenty-five 10 $\mu$ l injections were made into rapidly mixing (400 rpm) solution with 2 minute intervals between injections.

Initial concentration of perturbant placed in the calorimeter sample cell assumed a formula weight of 200 for each perturbant. The pH of each solution was checked after dissolution, but no adjustments of the pH were required. All  
30 experiments were performed at 30°C. Initial concentration of rhGh placed in the dropping syringe assumed a molecular weight of 20,000 for rhGh. The pH of each solution was checked after dissolution, but no adjustment of the pH was required.

The heats of reaction were determined by integration of the observed peaks. To correct for heat of mixing and dilution, a control experiment was also performed under identical conditions where aliquots of the test perturbant or rhGh were added to buffer solution only. The sum total of the heat evolved was plotted against the total perturbant concentration to produce the isotherm from which the association constant ( $K_A$ , M), enthalpy change ( $\Delta H$ , kcal/mol), entropy change ( $\Delta S$  (eu)), and N, and the stoichiometry of perturbant molecules complexed per equivalent of complexed supramolecular complex, were determined by curve-fitting the binding isotherm against the binding equation described for perturbant complexing in a supramolecular complex possessing one set of independent perturbant complexing sites. The data were deconvoluted using the nonlinear least squares algorithm supplied in the software of the manufacturer.

Results are illustrated in Table 11 below.

15

Table - 11					
Isothermal Titration Calorimetry of rhGH at pH 7.5 and 4.0 with Different Perturbants					
Perturbant	rhGh (mM)	$K_D$ (M)	$\Delta H$ (kcal/mol)	$\Delta S$ (eu)	N
<b>pH 7.5</b>					
A at 0.25mM	1.0	$9.88 \times 10^{-5}$	+ 1.4	+ 23.5	7.0
B at 0.25mM	1.0	$1.11 \times 10^{-6}$	+ 2.1	+ 35.0	0.7
C at 0.25mM	1.0	$1.11 \times 10^{-9}$	+ 0.8	+44.0	10.0
<b>pH 4.0</b>					
A at 0.25mM	1.0	$7.81 \times 10^{-5}$	-1.5	- 5.6	2.3
B at 0.25mM	1.0	$1.61 \times 10^{-9}$	-35.6	-90.0	155.9
C at 0.25mM	1.0	$2.67 \times 10^{-8}$	-1.2	-30.0	122.0

25  
30  
A = cyclohexanoyl chloride modified L-tyrosine  
B = salicyloyl modified L-phenylalanine  
C = phenylsulfonyl-para-aminobenzoic acid

The positive  $\Delta S$  values at pH 7.5 indicate that complexing at this pH results in structural change.

5     **Examples 46 and 47 - Pancreatin Inhibition Assay with  $\alpha$ -Interferon and Perturbants**

10     The assay for pancreatin activity was prepared as follows: 0.1mL of a stock solution of  $\alpha$ -interferon (9.1mg/mL, 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2) (Schering-Plough Corp.) was added to 2.5mL of either phenylsulfonyl-para-aminobenzoic acid perturbant (46) or cyclohexanoylphenylglycine perturbant (47) (200mg/mL) in 5mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0. Incubation was carried out at 37°C for 30 and 60 minutes following the addition of 0.1mL of USP pancreatin (20mg/mL) (Sigma Chemical Co.) 0.1mL aliquots were withdrawn at those times points. Enzyme reactions were stopped by the addition of protease inhibitors (Aprotinin and Bowman-Birk Inhibitor (BBI), each at 2mg/mL) and were diluted five-fold to  
15     quantitate  $\alpha$ -interferon left intact. A reverse phase HPLC method using a Butyl C-4 cartridge (3.0x0.46cm, Rainin) and employing gradient elution between 0.1% TFA/water and 90% ACN in 0.1% TFA coupled with UV detection at 220nm was used for separating and quantitating  $\alpha$ -interferon. The  $\alpha$ -interferon at 0 minutes was quantitated from an aliquot prior to the addition of pancreatin  
20     and was taken to be 100%.

Results are illustrated in Figure 27.

25     Examples 46 and 47 illustrate that both supramolecular complexes resisted enzymatic degradation. However, in additional testing no correlation was shown between the enzyme inhibitors potency and the ability to deliver drug.

**Example 48 - DSC of Heparin at pH 5.0**

30     DSC thermograms of heparin at pH 5.0 were conducted according to the method of Example 11 using pH, GuHCl, and ionic strength as perturbants.

Thermograms were corrected by subtraction of a heparin .05M NaCl - phosphate buffer blank, but an individual blank was not used for each NaCl concentration.

Results are illustrated in Tables 12-14 below and in Figure 28.

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10

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TABLE - 12			
Effects of pH on the DSC Spectrum of 20 $\mu$ g/ml Heparin in 50 mM Phosphate Buffer			
	T <sub>m</sub> (C <sub>p,max</sub> )	$\Delta H$ (kcal/mol)	$\Delta H_{vh}$ (kcal/mol)
pH 6.0	62.5	232.1	13.8
pH 6.5 (a) (b)	62.7 71.8	213.9 751.9	56.8
pH 7.0 (a) (b)	47.1 72.9	187.1 136.4	27.6
pH 7.5	66.2	499.4	83.8

(a) = a domain

(b) = b domain

20

25

30

TABLE - 13			
Effects of 10M Guanidine Hydrochloride in 50mM Phosphate Buffer on the DSC Spectrum of Heparin			
	T <sub>m</sub> (C <sub>p,max</sub> )	$\Delta H$ (kcal/mol)	$\Delta H_{vh}$ (kcal/mol)
heparin	67.2	499.4	83.8
heparin + 0.5M GuHCl	50.5	287.3	170.9
heparin + 1.0M GuHCl	60.5	415.0	97.1
heparin + 1.5M GuHCl	---	1716.5	24.3
heparin + 2.0M GuHCl	---	2533.7	19.2

TABLE - 14				
Effect of Ionic Strength on the DSC Spectrum of 20 $\mu$ g/ml of Heparin in 50mM Phosphate Buffer pH 7.0				
	T <sub>m</sub> (C <sub>p,max</sub> )		$\Delta H$ (kcal/mol)	$\Delta H_{WH}$ (kcal/mol)
0.0 M NaCl	47.1	187.1	72.9	136.4
0.25M NaCl	46.1	0.112	not present	
0.50M NaCl	41.6	0.094	not present	
0.75M NaCl	27.5	0.00	not present	
1.0 M NaCl	no transition observed			

These data indicate that non-proteinaceous active agents are able to change conformation in response to a perturbant.

#### 15 **Example 49 - Column Chromatography of Heparin and Perturbants**

The following materials were used:

##### Column:

10mm x 30cm, low pressure, glass column from Pharmacia w/adjustable bed volume. The bed volume used was 22 cm at a pressure of 0.8 Mpa.

##### Packing:

Heparin covalently bonded to Sepharose CL-6B with no linker molecule.

Sepharose fractionation range: 10, 000 - 4,000,000.

The density of heparin was 2mg/cc as per Pharmacia Q.C. Department.

##### Conditions:

The mobil phase was 67mM phosphate buffer, pH7.4.

The flow rate was 1.5mL/min isocratic.

The run time was 45 minutes.

Sample detection was done with a Perkin Elmer refractive index detector.

Column integrity was confirmed by injecting pr tamine and observing a retention time greater than 1 hour. Void volume was determined by injecting water and measuring time of elution.

Each of the perturbants of Table 15 below (5mg) was independently dissolved in 1mL of mobil phase and injected (100ul) into the column. Time of elution was measured. K' value was determined by using the following equation (as per USP):

$$K' = (\text{Ret. time Carrier} / \text{Ret. time Water}) - 1$$

The results were compared between each perturbant as well as their respective *in vivo* performance in Figure 29. K' (the degree of retardation) values in the figure have been corrected by subtraction of the K' value determined from the sepharose column from the K' value determined from the heparin-sepharose column.

15	Table - 15	
	PERTURBANTS	
	cyclohexylidenebutyric acid (2)-Na salt	#1
	cylcohexanebutyroyl (2-) aminobutyric acid (4)	#2
	phenylacetyl-para-aminobutyric acid	#3
20	ortho-methylcyclohexanoyl - aminobutyric acid (4)	#4
	phenylacetyl-amino hexanoic acid (6-)	#5
	cinnamoyl-para-aminophenylbutyric acid	#6
	cyclohexanebutyroyl (2-) - para-aminophenylbutyric acid	#7
	hydrocinnamoyl - para-aminophenylbutyric acid	#8
25	cyclohexanebutyroyl (2-)-leu-leu	#9
	cyclohexanebutyroyl (2-)-gly	#10

### 30 Example 50 - Oral Administration of Heparin to Rats

Rats were dosed with the dosing solutions of Table 16 below according to the procedure of Example 2. Blood was collected, and activated partial

thromboplastin time (APTT) was performed as described in Henry, J.B., *Clinical Diagnosis and Management by Laboratory Methods*, W.B. Saunders, 1979.

Results are illustrated in Figures 29 and 30.

Figure 29 illustrates that as predicted in the model, the greater the binding to heparin the greater the elevation of APTT. The data suggests that at  $K'$  values below 0.2, activity is likely to be poor. At  $K'$  values  $> 0.2$ , activity will be significant.

The data indicate a correlation between the retardation by the heparin sepharose column relative to just a sepharose column and the increased *in vivo* activity as measured by elevation of APTT. Notably protamine, which binds most strongly to heparin, has no oral bioavailability ( $K' = 3.68$ ). This indicates that balancing binding strength and conformational changes with the ability to dissociate will optimize the full complement of biological activity of the drug.

15

Table - 16	
HEPARIN/PERTURBANTS DOSING SCHEDULES	
	Solution 1 = cinnamoyl-para-aminophenylbutyric acid pH 7.5, N = 5
	Solution 2 = cinnamoyl-para-aminophenylbutyric acid (300 mg/kg) + Heparin (100 mg/kg) in propylene glycol/water (1:1, pH 7.4)
20	Solution 3 - Heparin (100 mg/kg, pH 7.4, N = 5)
	Solution 4 = hydrocinnamoyl-para-aminophenylbutyric acid (300 mg/kg) + Heparin (100 mg/kg) in propylene glycol/water (1:1, pH 7.4)

25

All patents, applications, test methods, and publications mentioned herein are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed disclosure. For example, oral drug delivery entails crossing from the lumen of the gastrointestinal tract to the blood. This occurs as a result of crossing several cellular lipid bilayers that separate these anatomical compartments. The complexation of the perturbant with the active agent and the change in conformation of the active agent creates a supramolecular complex having physicochemical properties, such as, for example, solubility and conformation in space, which are different than those

30

of either the perturbant or the active agent alone. This suggests that one can take advantage of this property to cross other membranes such as the blood-brain barrier, and ophthalmic, vaginal, rectal, and the like membranes. All such modifications are within the full extended scope of the appended claims.



**IN THE CLAIMS:**

- 1           1.     A method for transporting a biologically active agent across a  
2 cellular membrane or a lipid bilayer, said method comprising:
- 3           (a)     providing a biologically active agent which can exist in a native  
4 conformational state, a denatured conformational state, and an intermediate  
5 conformational state which is reversible to said native state and is  
6 conformationally between said native and denatured states;
- 7           (b)     exposing said biologically active agent to a complexing perturbant  
8 to reversibly transform said biologically active agent to said intermediate state  
9 and to form a transportable supramolecular complex,  
10                   said perturbant having a molecular weight between about  
11                   150 to about 600 daltons, and having at least one hydrophilic  
12                   moiety and at least one hydrophobic moiety,  
13                   said supramolecular complex comprising said perturbant non-  
14                   covalently complexed with said biologically active agent, and  
15                   said biologically active agent not forming a microsphere with  
16                   said perturbant; and
- 17           (c)     exposing said membrane or bilayer to said supramolecular complex,  
18 to transport said biologically active agent across said membrane or bilayer.
- 1           2.     A method as defined in claim 1, further comprising  
2                   (d)     removing said perturbant from said supramolecular complex  
3 to transform said biologically active agent to said native state.
- 1           3.     A method as defined in claim 2, wherein step (d) comprises diluting  
2 said supramolecular complex.
- 1           4.     A method as defined in claim 1, wherein said intermediate state has  
2 a  $\Delta G$  ranging from about -20 kcal/mole to about 20 kcal/moles.
- 1           5.     A method as defined in claim 1, wherein said biologically active  
2 agent is selected from the group consisting of a peptide, a

3 mucoo polysaccharide, a carbohydrate, a lipid, a pesticide, or any combination  
4 of the foregoing.

1 6. A method as defined in claim 5, wherein said biologically-active  
2 agent is selected from the group consisting of human growth hormone, bovine  
3 growth hormone, growth hormone-releasing hormone, an interferon, interleukin-  
4 II, insulin, heparin, calcitonin, erythropoietin, atrial natriuretic factor, an antigen,  
5 a monoclonal antibody, somatostatin, adrenocorticotropin, gonadotropin  
6 releasing hormone, oxytocin, vasopressin, cromolyn sodium, vancomycin,  
7 desferrioxamine (DFO), or any combination of any of the foregoing.

1 7. A method as defined in claim 1, wherein said perturbant is selected  
2 from the group consisting of  
3 (a) a proteinoid;  
4 (b) an acylated amino acid;  
5 (c) an acylated poly amino acid;  
6 (d) a sulfonated amino acid;  
7 (e) a sulfonated poly amino acid;  
8 (f) an acylated aldehyde of an amino acid;  
9 (g) an acylated ketone of an amino acid;  
10 (h) an acylated aldehyde of a poly amino acid;  
11 (i) an acylated ketone of a poly amino acid; and  
12 (j) a carboxylic acid having the formula

13 
$$R-CO_2H$$

14 wherein R is C<sub>1</sub> to C<sub>24</sub> alkyl, C<sub>2</sub> to C<sub>24</sub> alkenyl, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to  
15 C<sub>10</sub> cycloalkenyl, phenyl, naphthyl, (C<sub>1</sub> to C<sub>10</sub> alkyl)phenyl, (C<sub>2</sub> to C<sub>10</sub>  
16 alkenyl)phenyl, (C<sub>1</sub> to C<sub>10</sub> alkyl)naphthyl, (C<sub>2</sub> to C<sub>10</sub> alkenyl)naphthyl, phenyl(C<sub>1</sub>  
17 to C<sub>10</sub> alkyl), phenyl(C<sub>2</sub> to C<sub>10</sub> alkenyl), naphthyl(C<sub>1</sub> to C<sub>10</sub> alkyl) and naphthyl(C<sub>2</sub>  
18 to C<sub>10</sub> alkenyl);

19 R being optionally substituted with C<sub>1</sub> to C<sub>10</sub> alkyl, C<sub>2</sub> to C<sub>10</sub> alkenyl, C<sub>1</sub>  
20 to C<sub>4</sub> alkoxy, -OH, -SH, -CO<sub>2</sub>R<sup>1</sup>, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to C<sub>10</sub> cycloalkenyl,  
21 heterocyclic having 3-10 ring atoms wherein the hetero atom is one or more

22 at ms of N, O, S or any c mbinati n thereof, aryl, (C<sub>1</sub> to C<sub>10</sub> alkyl)aryl, aryl(C<sub>1</sub>  
23 t C<sub>10</sub>)alkyl, r any c mbination thereof;  
24 R being optionally interrupted by oxygen, nitrogen, sulfur, or any  
25 combination thereof; and  
26 R<sup>1</sup> is hydrogen, C<sub>1</sub> to C<sub>4</sub> alkyl or C<sub>2</sub> to C<sub>4</sub> alkenyl; or  
27 a salt thereof.

28

1 8. A method for preparing an orally administrable biologically active  
2 agent, said method comprising:  
3 (a) providing a biologically active agent which can exist in a native  
4 conformational state, a denatured conformational state, and an intermediate  
5 conformational state which is reversible to said native state and is  
6 conformationally between said native and denatured states; and  
7 (b) exposing said biologically active agent to a complexing perturbant  
8 to reversibility transform said biologically active agent to said intermediate state  
9 and to form a transportable supramolecular complex,  
10 said perturbant having a molecular weight ranging from about 150  
11 to about 600 daltons, and having at least one hydrophilic moiety and at  
12 least one hydrophobic moiety,  
13 said supramolecular complex comprising said perturbant non-  
14 covalently complexed with said biologically active agent; and  
15 said biologically active agent not forming a microsphere with said  
16 perturbant.

1 9. A method as defined in claim 8, wherien said intermediate state has  
2  $\Delta G$  ranging from about -20 kcal/mole to about 20 kcal/moles.

1 10. A method as defined in claim 8, wherein said biologically active  
2 agent is selected from the group consisting of a peptide, a micropolysaccharide,  
3 a carbohydrate, a lipid, a pesticide, or any combination of the foregoing.

1 11. A m th d as defined in claim 10, wherein said biol gically-active  
2 agent is selected fr m the gr up consisting of human growth hormone, bovine

3 gr with hormone, growth hormone-releasing hormone, an interferon, interleukin-  
 4 ll, insulin, heparin, calcitonin, erythropoietin, atrial natriuretic factor, an antigen,  
 5 a monoclonal antibody, somatostatin, adrenocorticotropic hormone, gonadotropin  
 6 releasing hormone, oxytocin, vasopressin, cromolyn sodium, vancomycin,  
 7 desferrioxamine (DFO), or any combination of any of the foregoing.

1 12. A method as defined in claim 8, wherein said perturbant is selected  
 2 from the group consisting of

- 3 (a) a proteinoid;
- 4 (b) an acylated amino acid;
- 5 (c) an acylated poly amino acid;
- 6 (d) a sulfonated amino acid;
- 7 (e) a sulfonated poly amino acid;
- 8 (f) an acylated aldehyde of an amino acid;
- 9 (g) an acylated ketone of an amino acid;
- 10 (h) an acylated aldehyde of a poly amino acid;
- 11 (i) an acylated ketone of a poly amino acid; and
- 12 (j) a carboxylic acid having the formula



14 wherein R is C<sub>1</sub> to C<sub>24</sub> alkyl, C<sub>2</sub> to C<sub>24</sub> alkenyl, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to C<sub>10</sub>  
 15 cycloalkenyl, phenyl, naphthyl, (C<sub>1</sub> to C<sub>10</sub> alkyl)phenyl, (C<sub>2</sub> to C<sub>10</sub> alkenyl)phenyl,  
 16 (C<sub>1</sub> to C<sub>10</sub> alkyl)naphthyl, (C<sub>2</sub> to C<sub>10</sub> alkenyl)naphthyl, phenyl(C<sub>1</sub> to C<sub>10</sub> alkyl),  
 17 phenyl(C<sub>2</sub> to C<sub>10</sub> alkenyl), naphthyl(C<sub>1</sub> to C<sub>10</sub> alkyl) and naphthyl(C<sub>2</sub> to C<sub>10</sub>  
 18 alkenyl);

19 R being optionally substituted with C<sub>1</sub> to C<sub>10</sub> alkyl, C<sub>2</sub> to C<sub>10</sub> alkenyl, C<sub>1</sub>  
 20 to C<sub>4</sub> alkoxy, -OH, -SH, -CO<sub>2</sub>R<sup>1</sup>, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to C<sub>10</sub> cycloalkenyl,  
 21 heterocyclic having 3-10 ring atoms wherein the hetero atom is one or more  
 22 atoms of N, O, S or any combination thereof, aryl, (C<sub>1</sub> to C<sub>10</sub> alkyl)aryl, aryl(C<sub>1</sub>  
 23 to C<sub>10</sub>)alkyl, or any combination thereof;

24 R being optionally interrupted by oxygen, nitrogen, sulfur, or any  
 25 combination thereof; and

26 R<sup>1</sup> is hydrogen, C<sub>1</sub> to C<sub>4</sub> alkyl or C<sub>2</sub> to C<sub>4</sub> alkenyl; or

27                   a salt thereof.

1           13.    An oral delivery composition comprising a supramolecular complex  
2 comprising:  
3           (a)    a biologically active agent in an intermediate conformational state  
4 non-covalently complexed with  
5           (b)    a complexing perturbant having a molecular weight ranging from  
6 about 150 to about 600 and having at least one hydrophilic moiety and at least  
7 one hydrophobic moiety;  
8                   wherein said intermediate state is reversible to said native state and  
9 is conformationally between a native conformational and a denatured  
10 conformational state of said biologically active agent and said composition is not  
11 a microsphere.

1           14.    A method as defined in claim 13, wherein said biologically active  
2 agent is selected from the group consisting of a peptide, a micropolysaccharide,  
3 a carbohydrate, a lipid, a pesticide, or any combination of the foregoing.

1           15.    A method as defined in claim 14 wherein said biologically-active  
2 agent is selected from the group consisting of human growth hormone, bovine  
3 growth hormone, growth hormone-releasing hormone, an interferon, interleukin-  
4 II, insulin, heparin, calcitonin, erythropoietin, atrial naturetic factor, an antigen,  
5 a monoclonal antibody, somatostatin, adrenocorticotropin, gonadotropin  
6 releasing hormone, oxytocin, vasopressin, cromolyn sodium, vancomycin,  
7 desferrioxamine (DFO), or any combination of any of the foregoing.

1           16.    A method as defined in claim 13, wherein said perturbant is  
2 selected from the group consisting of  
3           (a)    a proteinoid;  
4           (b)    an acylated amino acid;  
5           (c)    an acylated p ly amino acid;  
6           (d)    a sulfonated amino acid;  
7           (e)    a sulfonated poly amino acid;

- 8 (f) an acylated aldehyde of an amino acid;  
 9 (g) an acylated ketone of an amino acid;  
 10 (h) an acylated aldehyde of a poly amino acid;  
 11 (i) an acylated ketone of a poly amino acid; and  
 12 (j) a carboxylic acid having the formula  
 13 
$$R-CO_2H$$

14 wherein R is C<sub>1</sub> to C<sub>24</sub> alkyl, C<sub>2</sub> to C<sub>24</sub> alkenyl, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to C<sub>10</sub>  
 15 cycloalkenyl, phenyl, naphthyl, (C<sub>1</sub> to C<sub>10</sub> alkyl)phenyl, (C<sub>2</sub> to C<sub>10</sub> alkenyl)phenyl,  
 16 (C<sub>1</sub> to C<sub>10</sub> alkyl)naphthyl, (C<sub>2</sub> to C<sub>10</sub> alkenyl)naphthyl, phenyl(C<sub>1</sub> to C<sub>10</sub> alkyl),  
 17 phenyl(C<sub>2</sub> to C<sub>10</sub> alkenyl), naphthyl(C<sub>1</sub> to C<sub>10</sub> alkyl) and naphthyl(C<sub>2</sub> to C<sub>10</sub>  
 18 alkenyl);

19 R being optionally substituted with C<sub>1</sub> to C<sub>10</sub> alkyl, C<sub>2</sub> to C<sub>10</sub> alkenyl, C<sub>1</sub>  
 20 to C<sub>4</sub> alkoxy, -OH, -SH, -CO<sub>2</sub>R<sup>1</sup>, C<sub>3</sub> to C<sub>10</sub> cycloalkyl, C<sub>3</sub> to C<sub>10</sub> cycloalkenyl,  
 21 heterocyclic having 3-10 ring atoms wherein the hetero atom is one or more  
 22 atoms of N, O, S or any combination thereof, aryl, (C<sub>1</sub> to C<sub>10</sub> alkyl)aryl, aryl(C<sub>1</sub>  
 23 to C<sub>10</sub>)alkyl, or any combination thereof;

24 R being optionally interrupted by oxygen, nitrogen, sulfur, or any  
 25 combination thereof; and

26 R<sup>1</sup> is hydrogen, C<sub>1</sub> to C<sub>4</sub> alkyl or C<sub>2</sub> to C<sub>4</sub> alkenyl; or  
 27 a salt thereof.

- 1 17. A dosage unit form comprising  
 2 (A) a composition as defined in claim 13; and  
 3 (B) (a) an excipient,  
 4 (b) a diluent,  
 5 (c) a disintegrant,  
 6 (d) a lubricant,  
 7 (e) a plasticizer,  
 8 (f) a colorant,  
 9 (g) a dosing vehicle, or  
 10 (h) any combination thereof.

1           18. A method for preparing an agent which is transportable across a  
2 cellular membrane or a lipid-bilayer and which is bioavailable after crossing said  
3 membrane or bilayer, said method comprising

4           (a) providing a biologically active agent which can exist in a native  
5 conformational state, a denatured conformational state, and an intermediate  
6 conformational state which is reversible to said native state and is  
7 conformationally between said native and denatured states;

8           (b) exposing said biologically active agent to a complexing perturbant  
9 to reversibly transform said biologically active agent to said intermediate state  
10 and to form a transportable supramolecular complex,

11           said perturbant having a molecular weight between about 150 and about  
12 600 daltons, and having at least one hydrophilic moiety and one hydrophilic  
13 moiety,

14           said supramolecular complex comprising said perturbant non-covalently  
15 complexed with said biologically active agent, and

16           said biologically active agent not forming a microsphere with said  
17 perturbant; and

18           (c) preparing a mimetic of said supramolecular complex.

1           19. A method as defined in claim 18, wherein said biologically active  
2 agent comprises a peptide and said mimetic comprises a peptide mimetic.

1           20. A method for preparing an agent which is transportable across a  
2 cellular membrane or a lipid-bilayer, and which is bioavailable after crossing said  
3 membrane or bilayer, said method comprising

4           (a) providing a biologically active agent which can exist in a native  
5 conformational state, a denatured conformational state, and an intermediate  
6 which is reversible to said native state and is conformationally between said  
7 native and denatured states;

8           (b) exposing said biologically active agent to a perturbant to reversibly  
9 transform said biologically active agent to said intermediate state; and

10           (c) preparing a mimetic of said intermediate state.

1           21. A method as defined in claim 20, wherein said perturbant  
2 comprises a pH changing agent, an ionic strength changing agent, or guanidine  
3 hydrochloride.

1           22. An oral delivery composition comprising a mimetic of the oral  
2 delivery composition prepared by the method of claim 8.

1           23. An oral delivery composition comprising a peptide mimetic of tan  
2 intermediate conformational state of a peptidic biologically active agent having  
3 a native state, a denatured state, and an intermediate state conformationally  
4 between said native and denatured states, wherein said intermediate state is  
5 reversible to said native state.



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FIG. 1

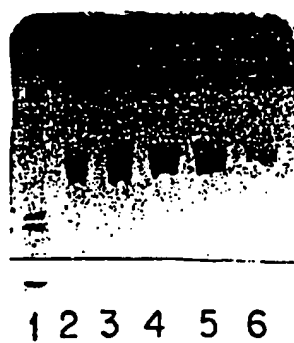
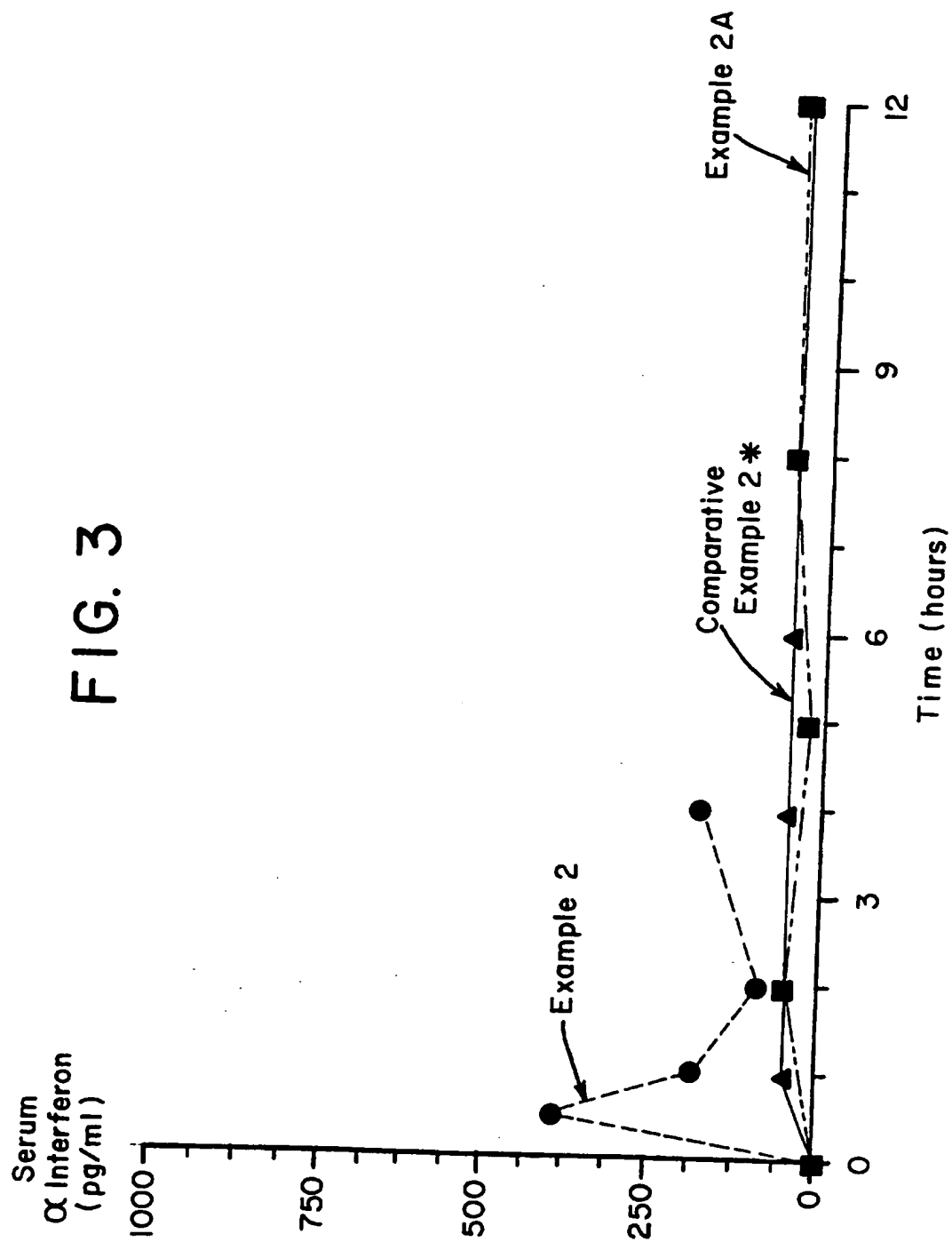


FIG. 2

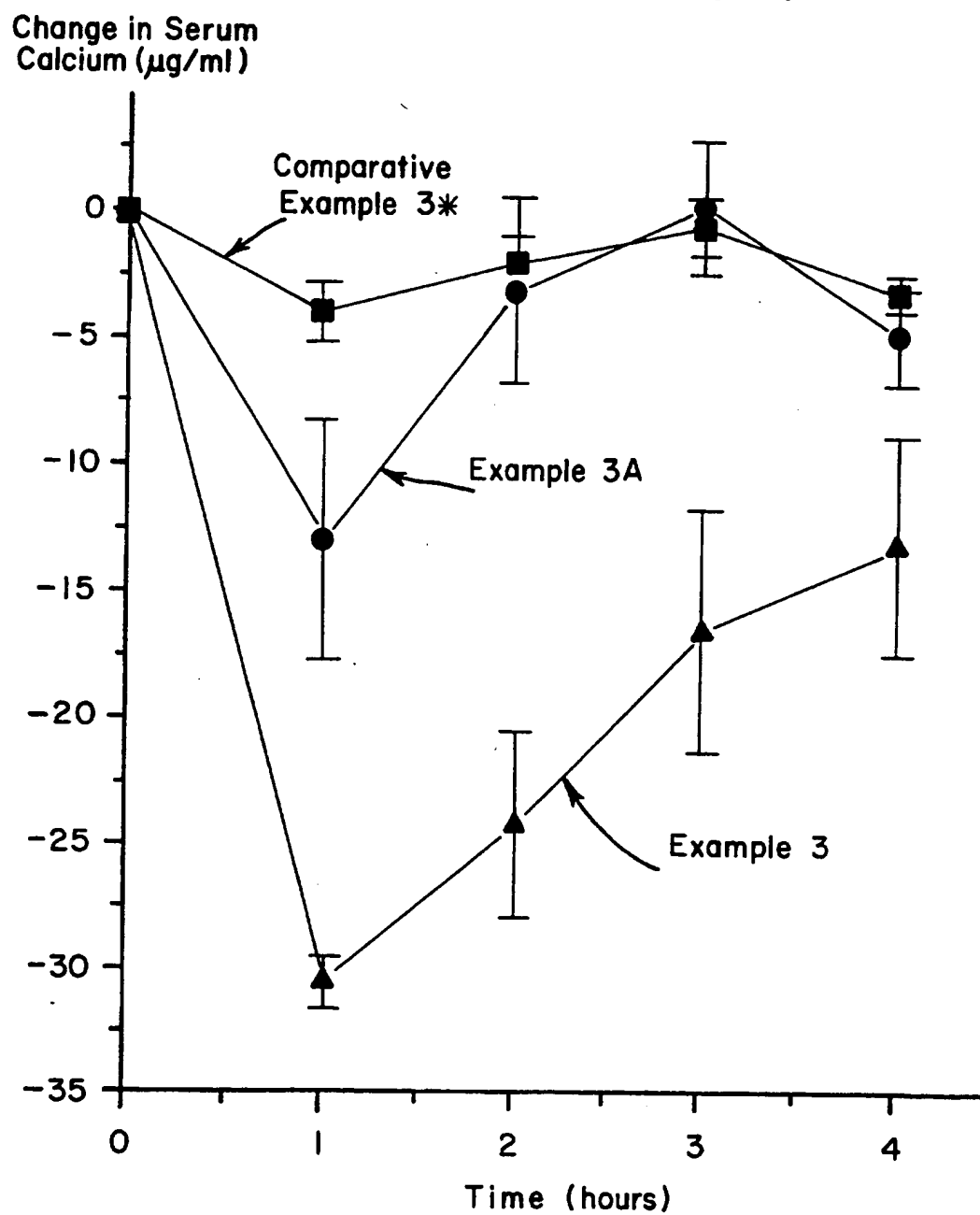


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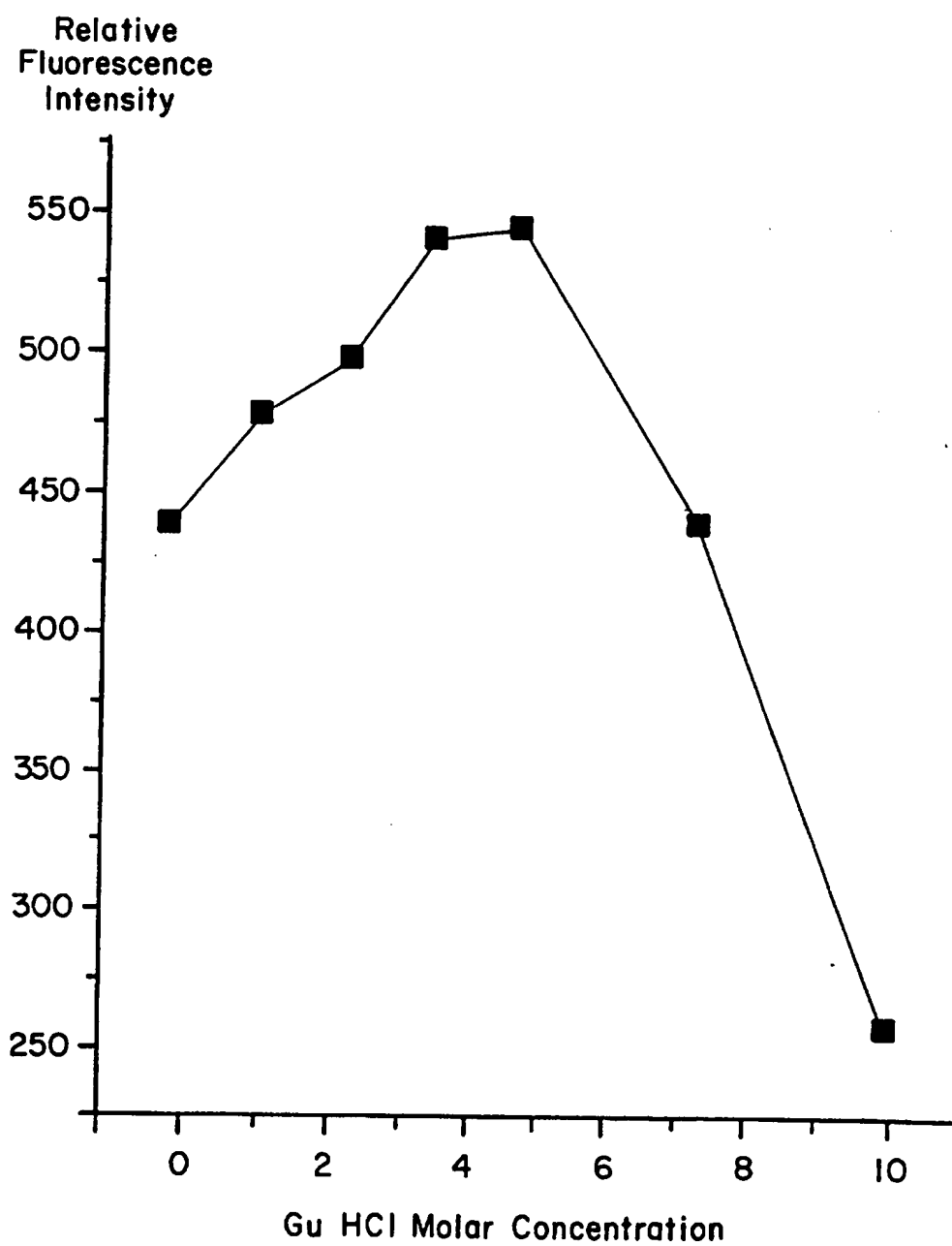
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FIG. 4



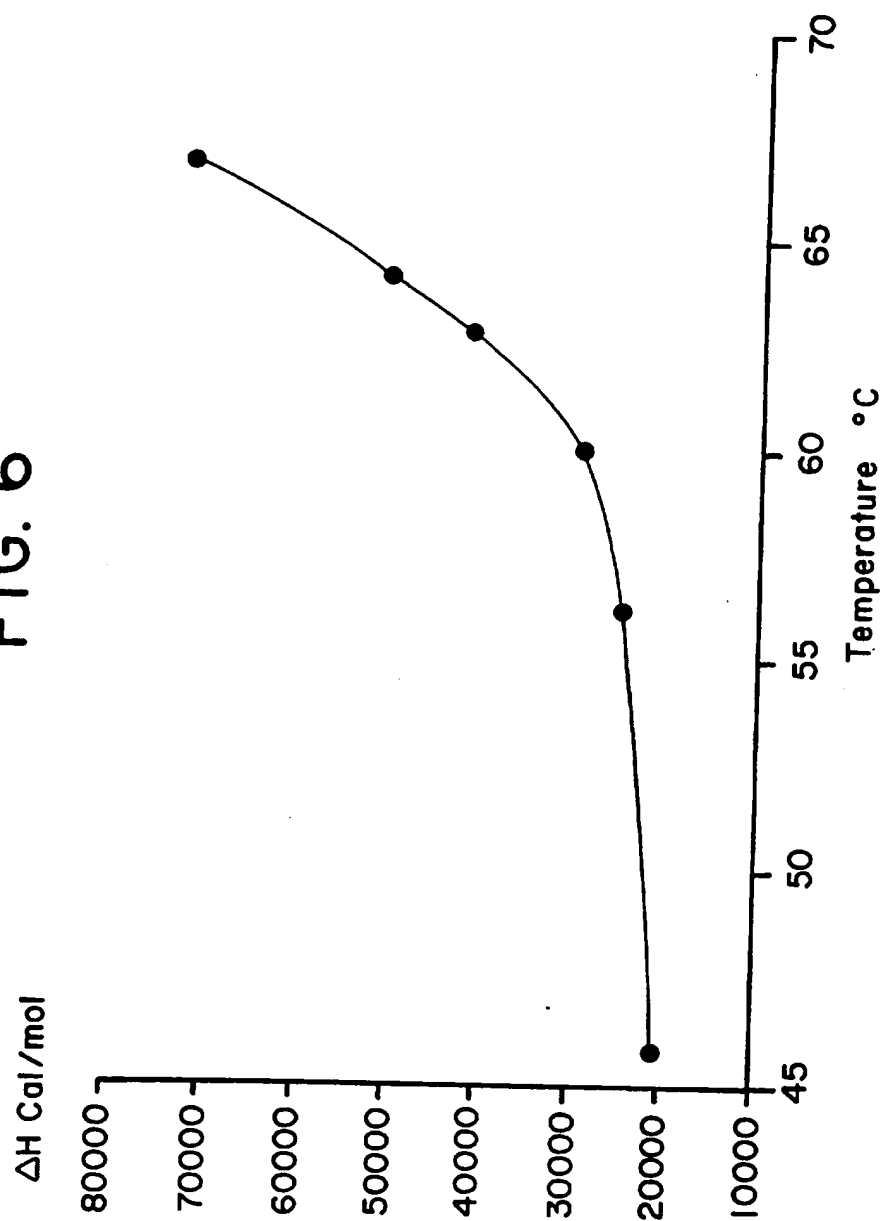
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FIG. 5



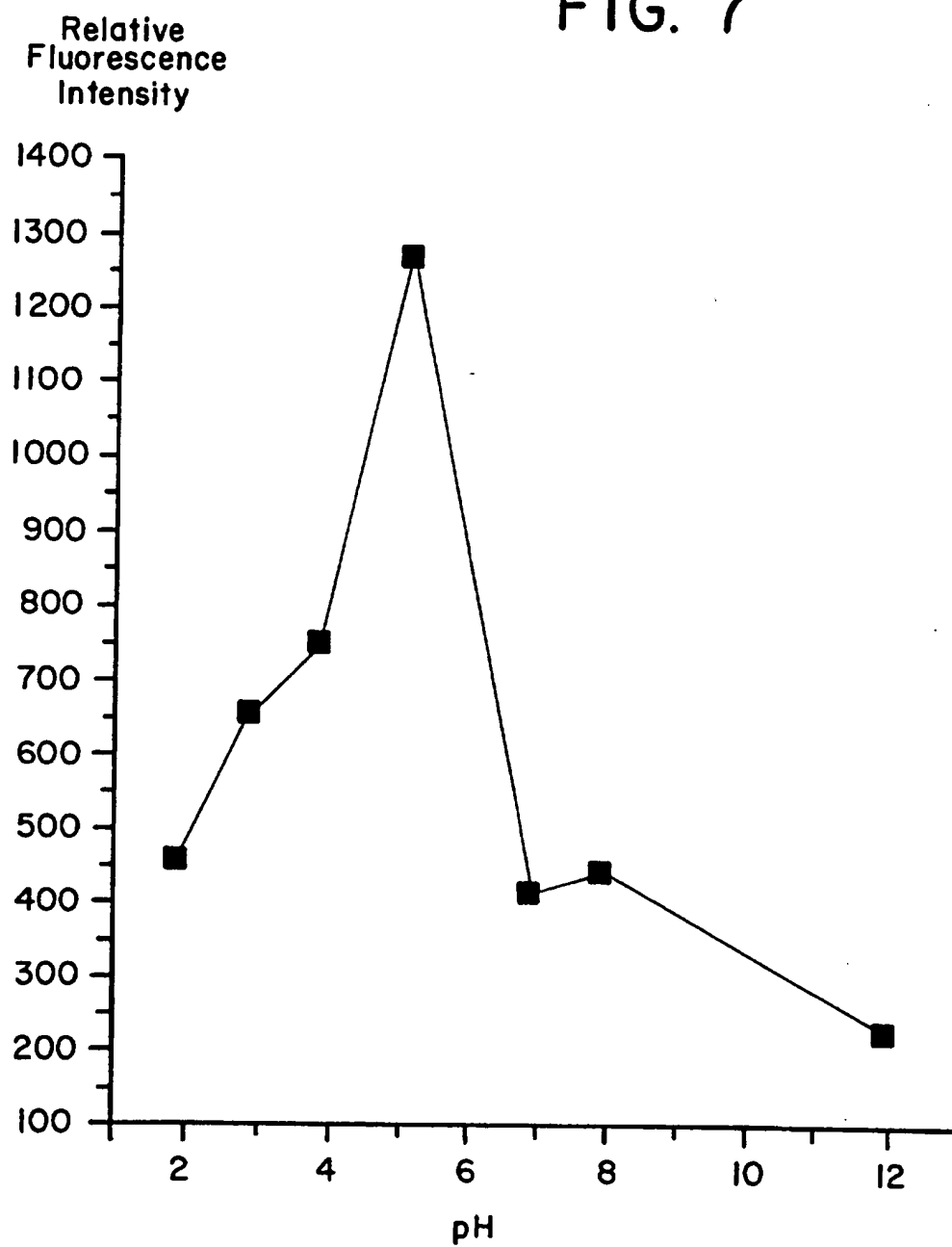
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FIG. 6



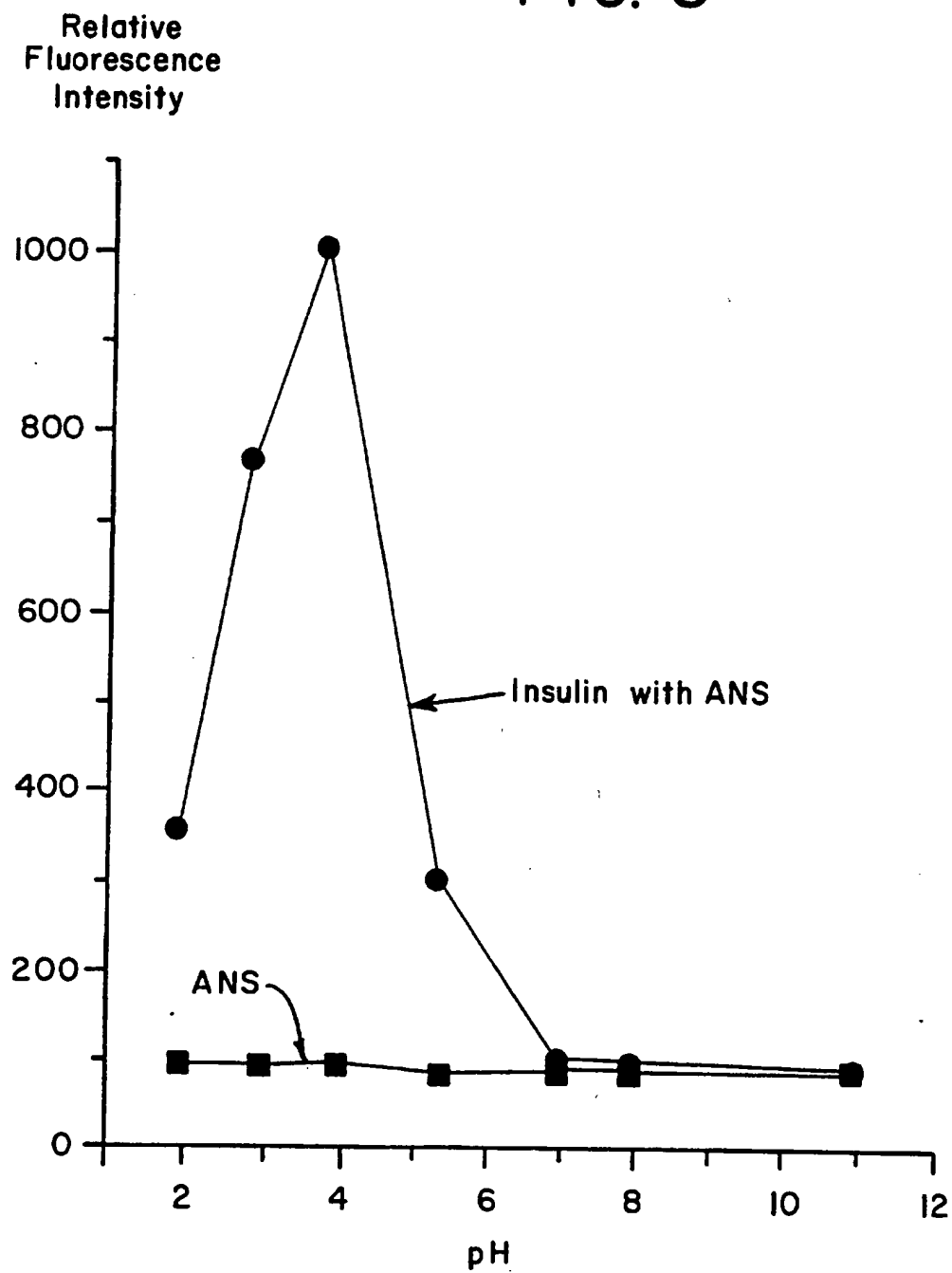
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FIG. 7



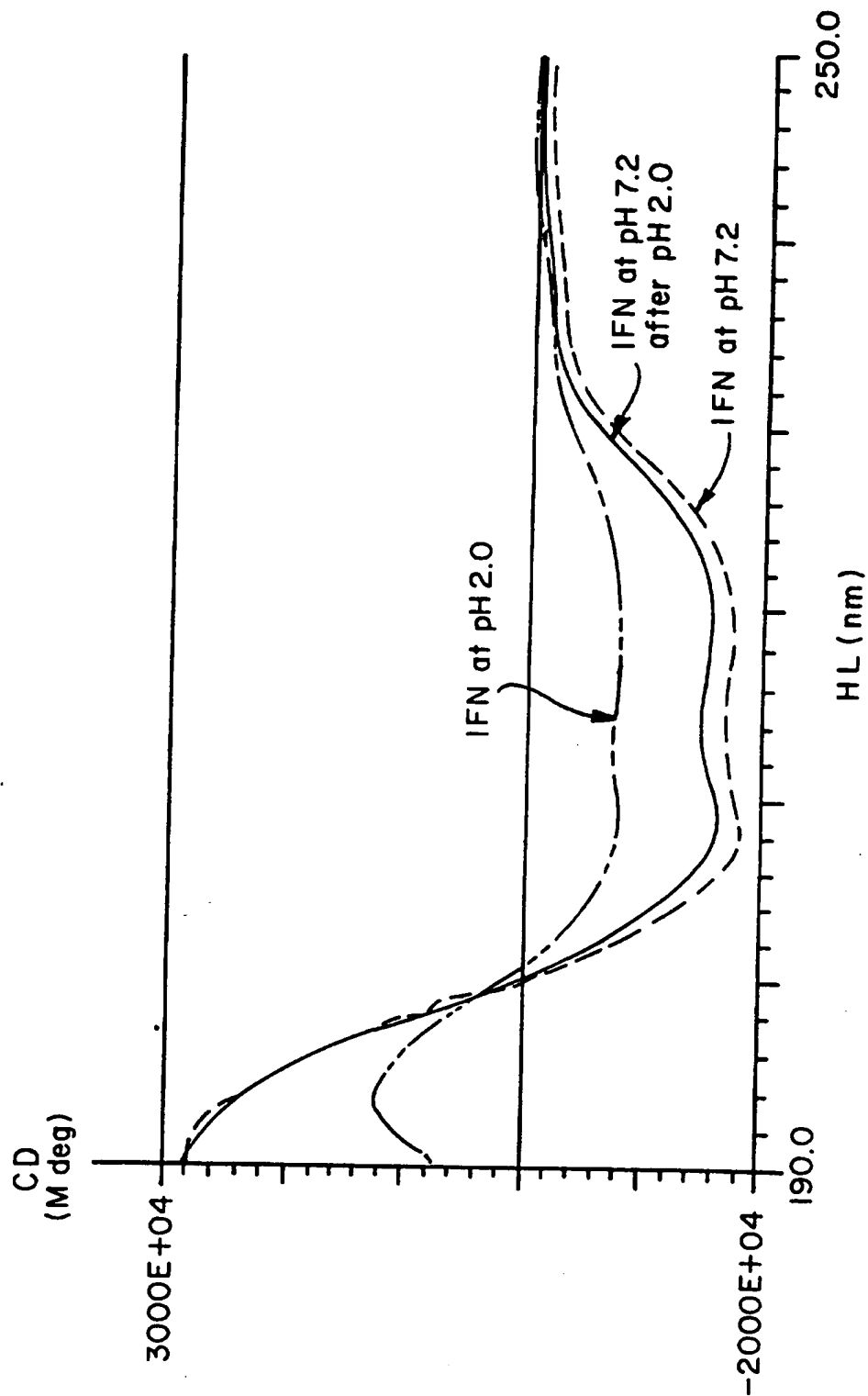
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FIG. 8



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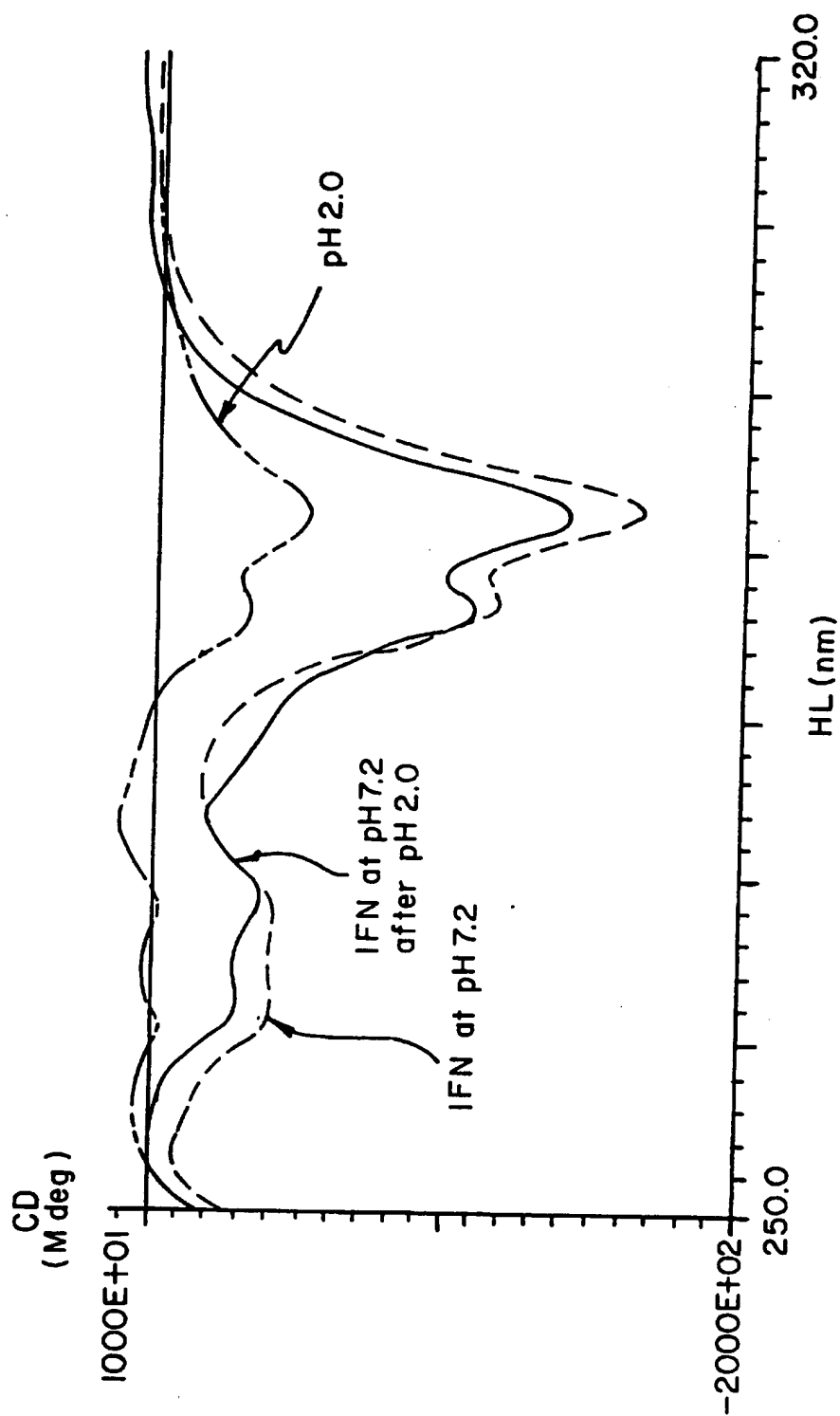
FIG. 9A



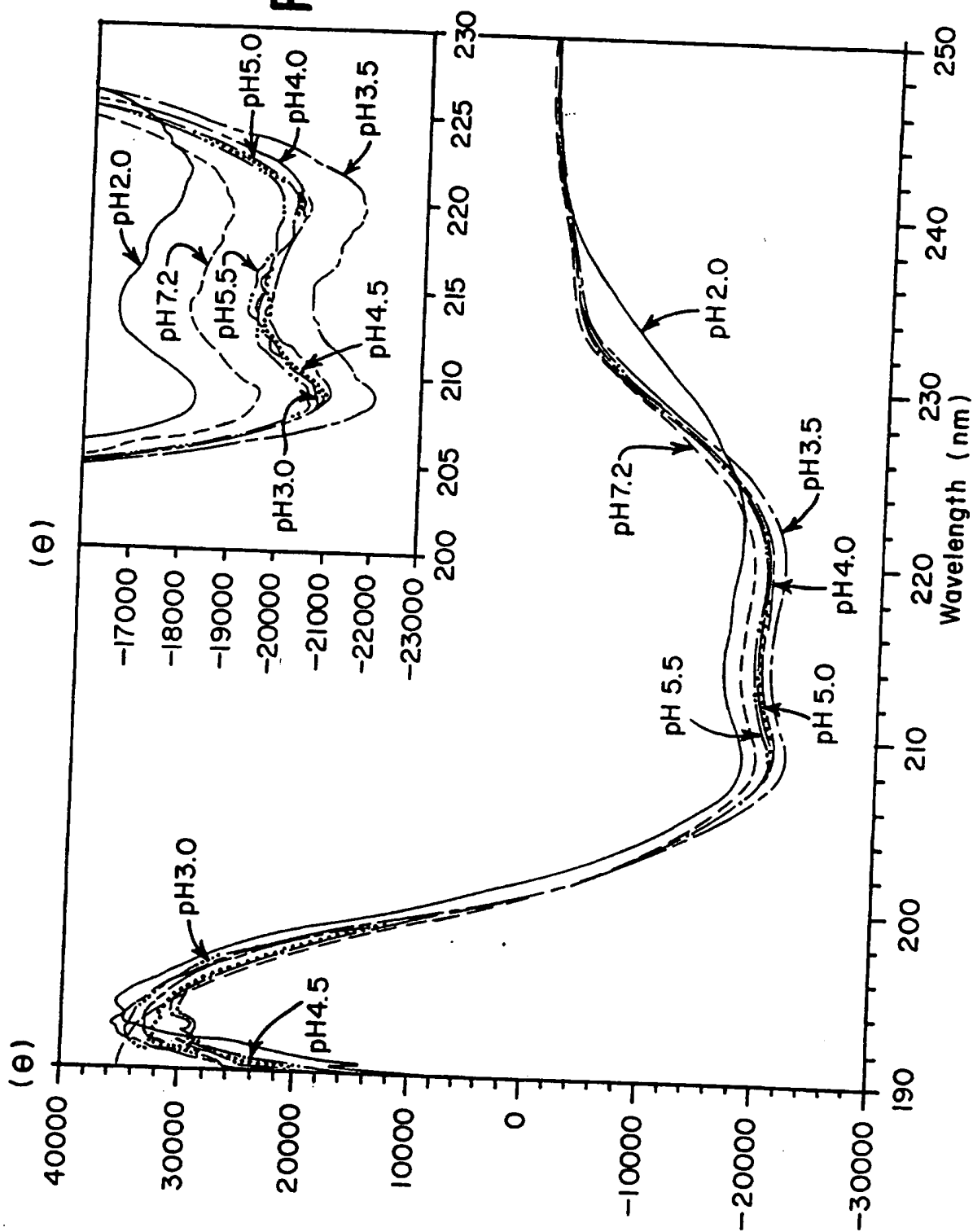


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FIG. 9B

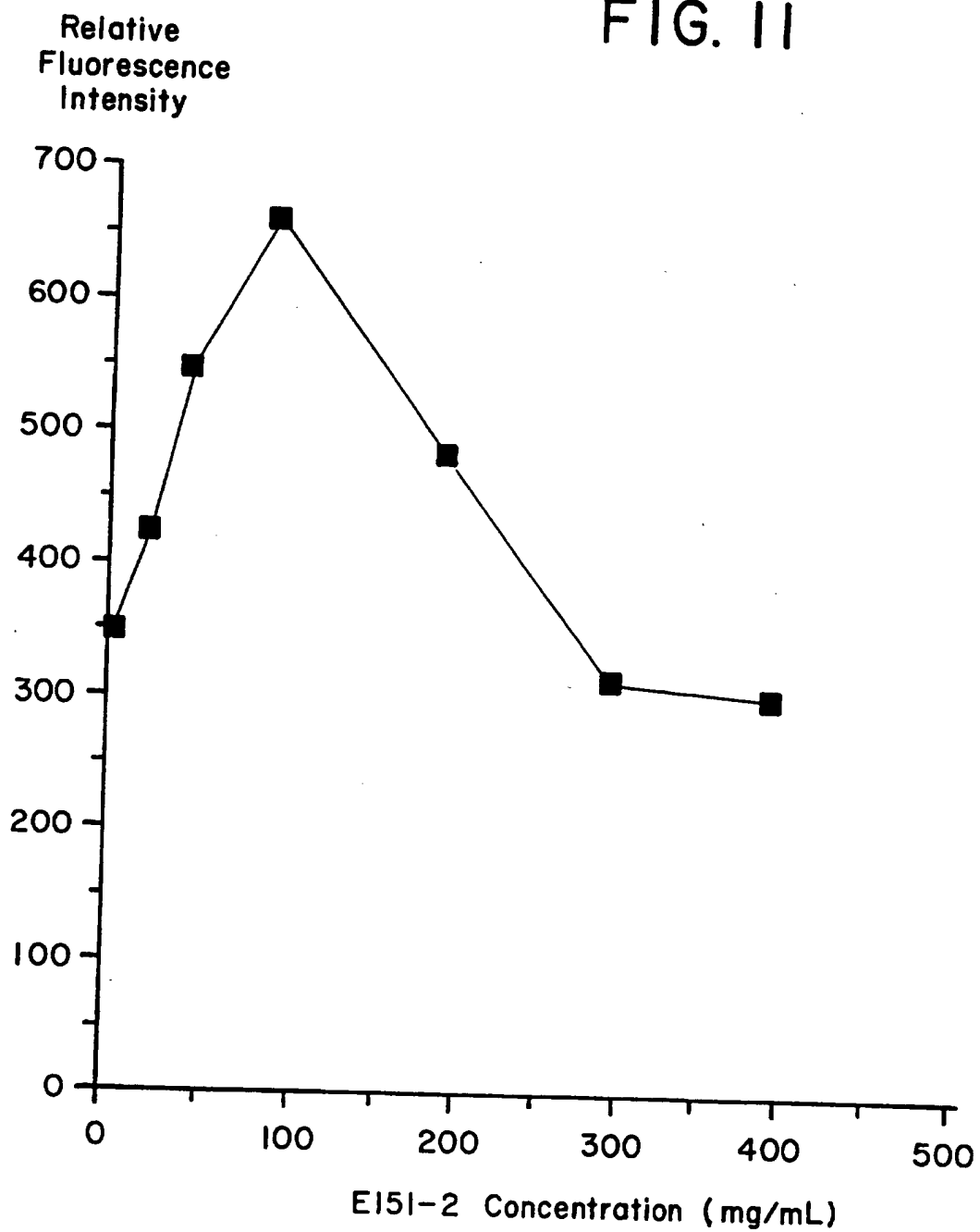


**FIG. 10**

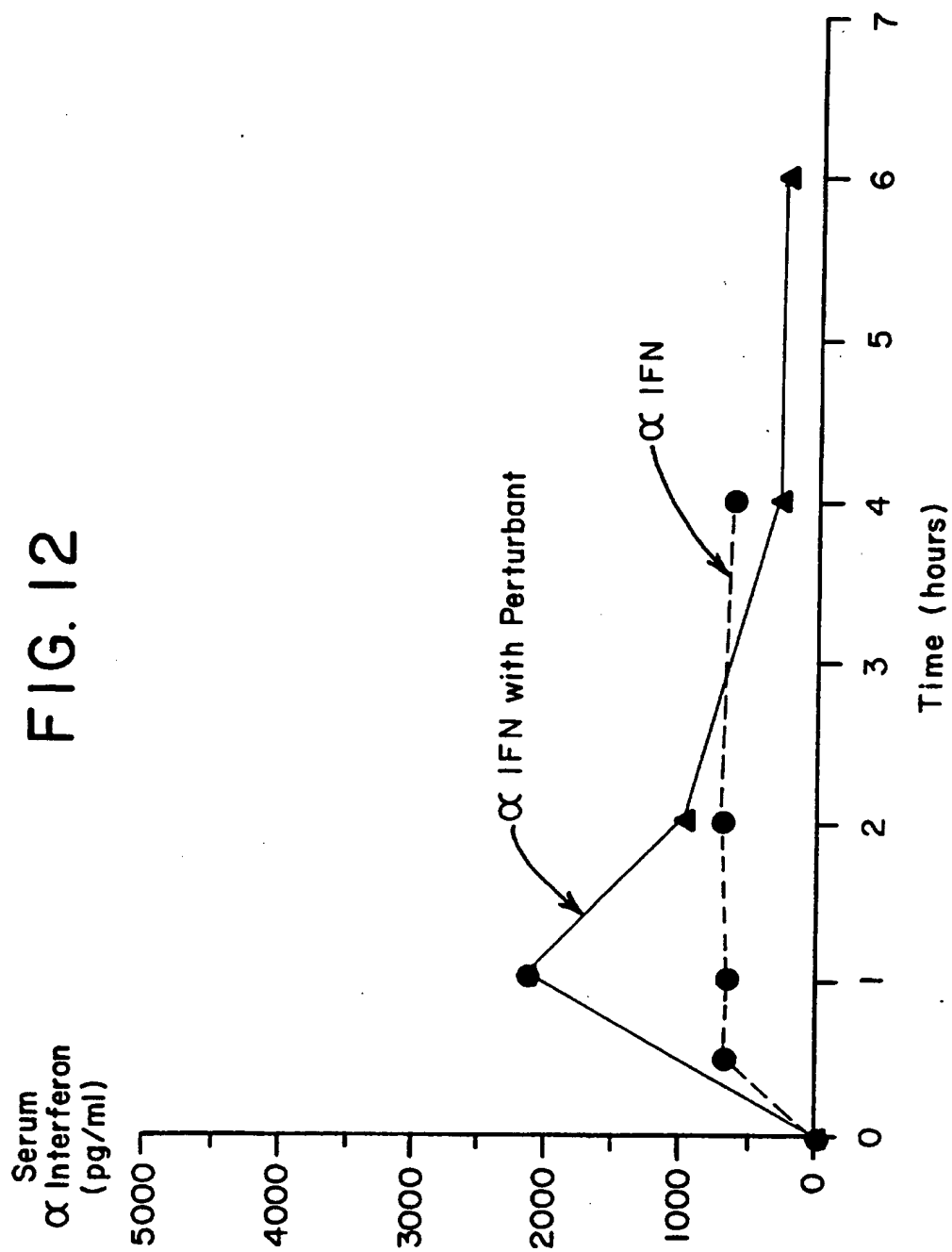


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FIG. 11

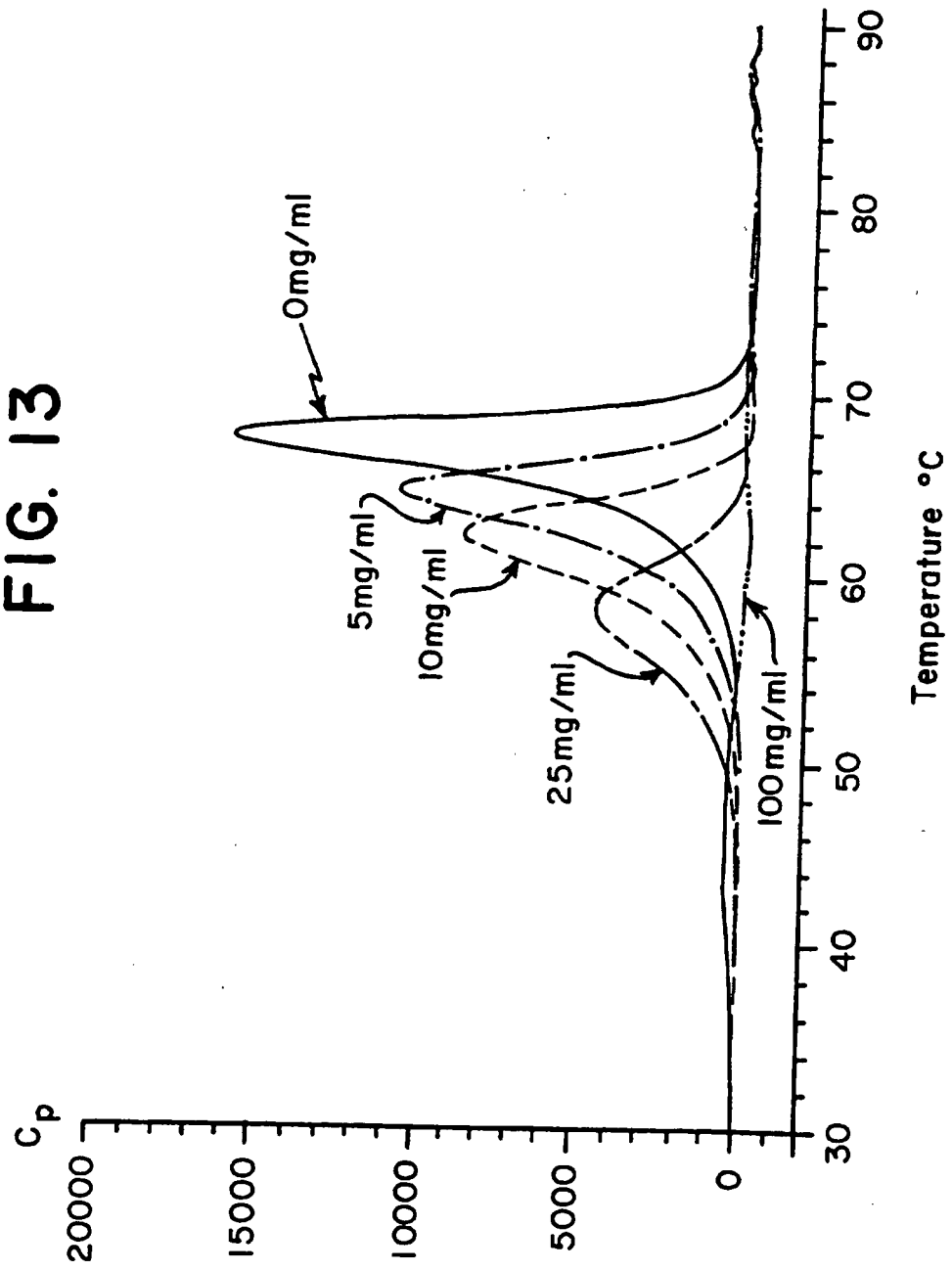


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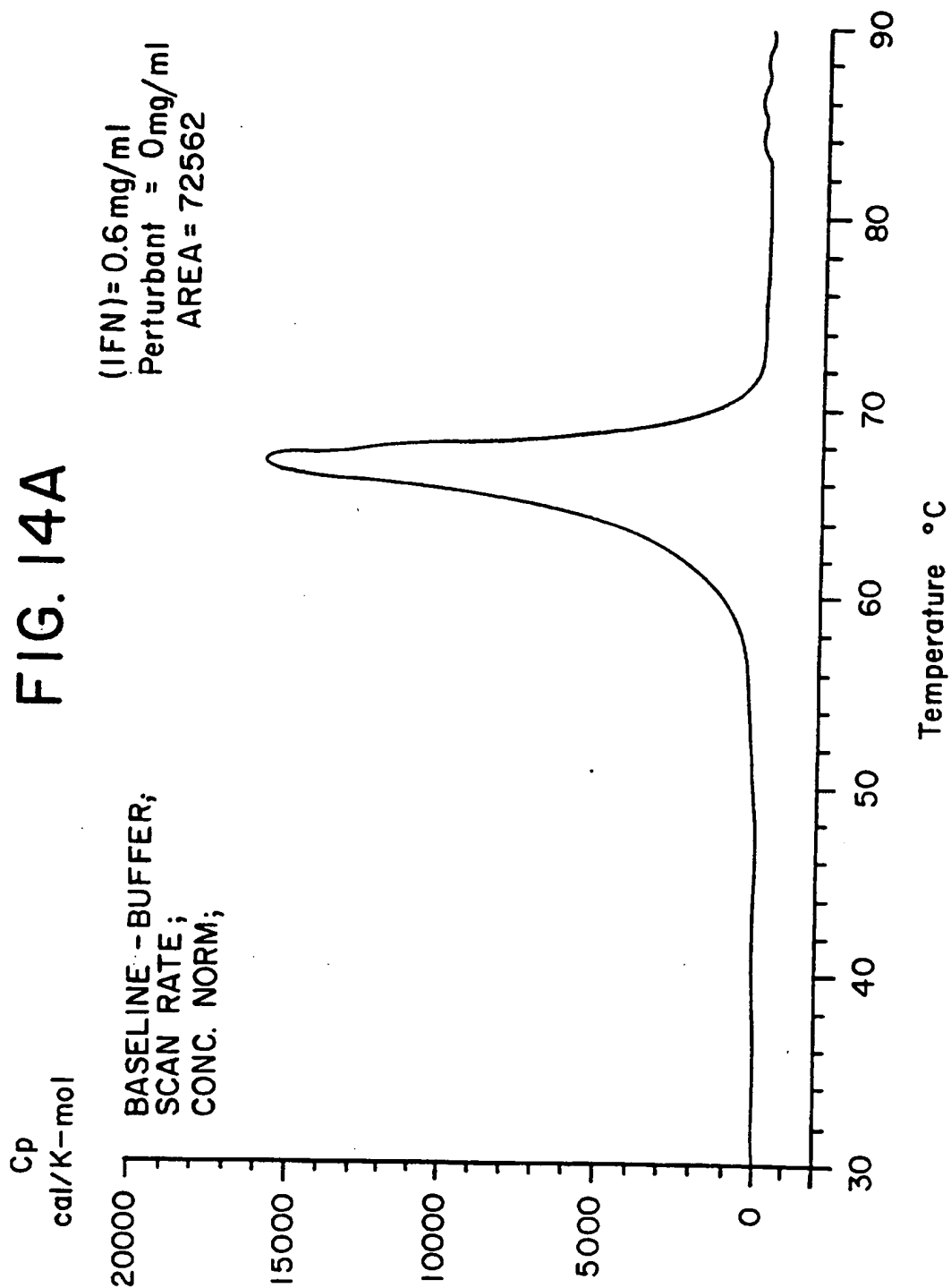


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FIG. 13

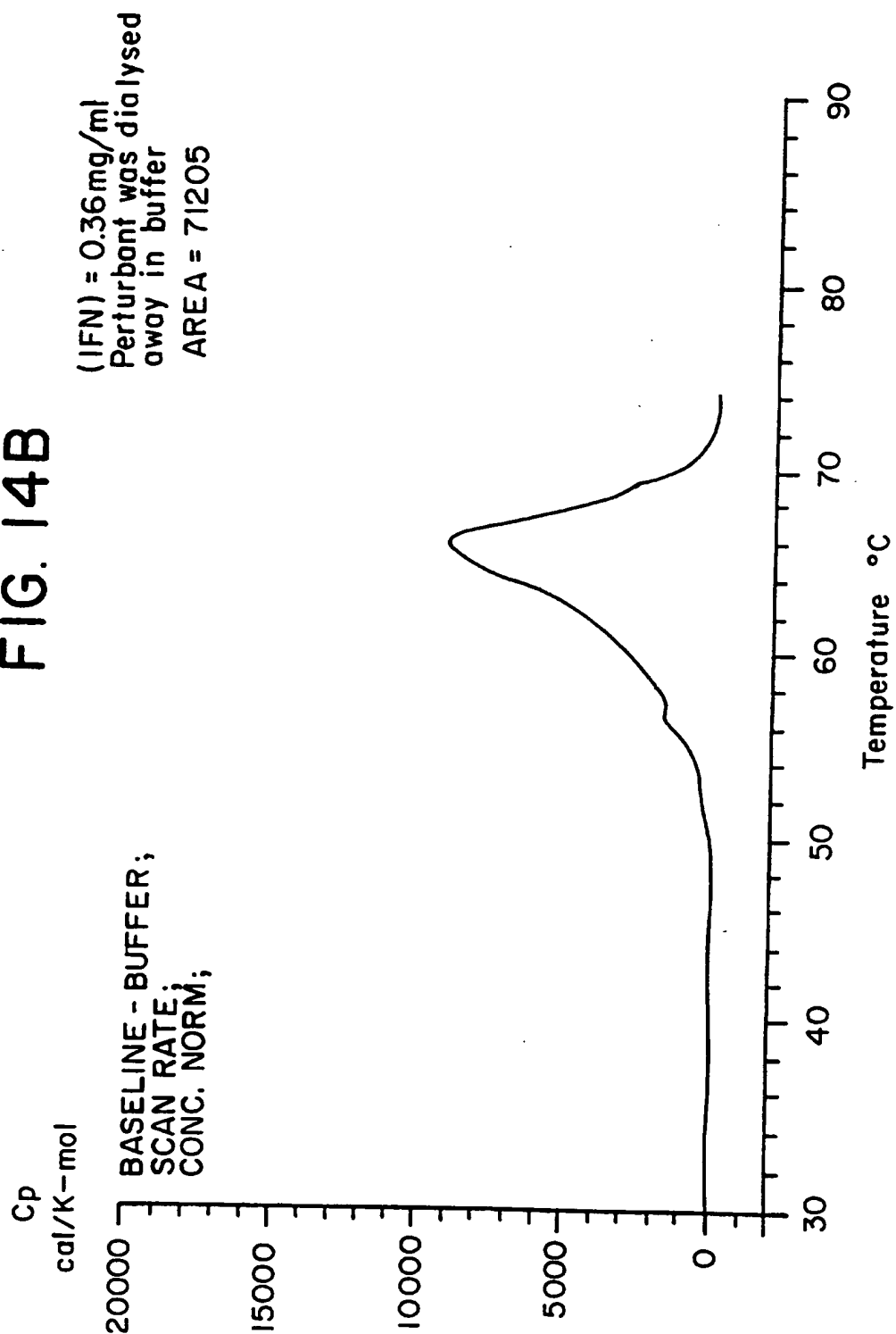


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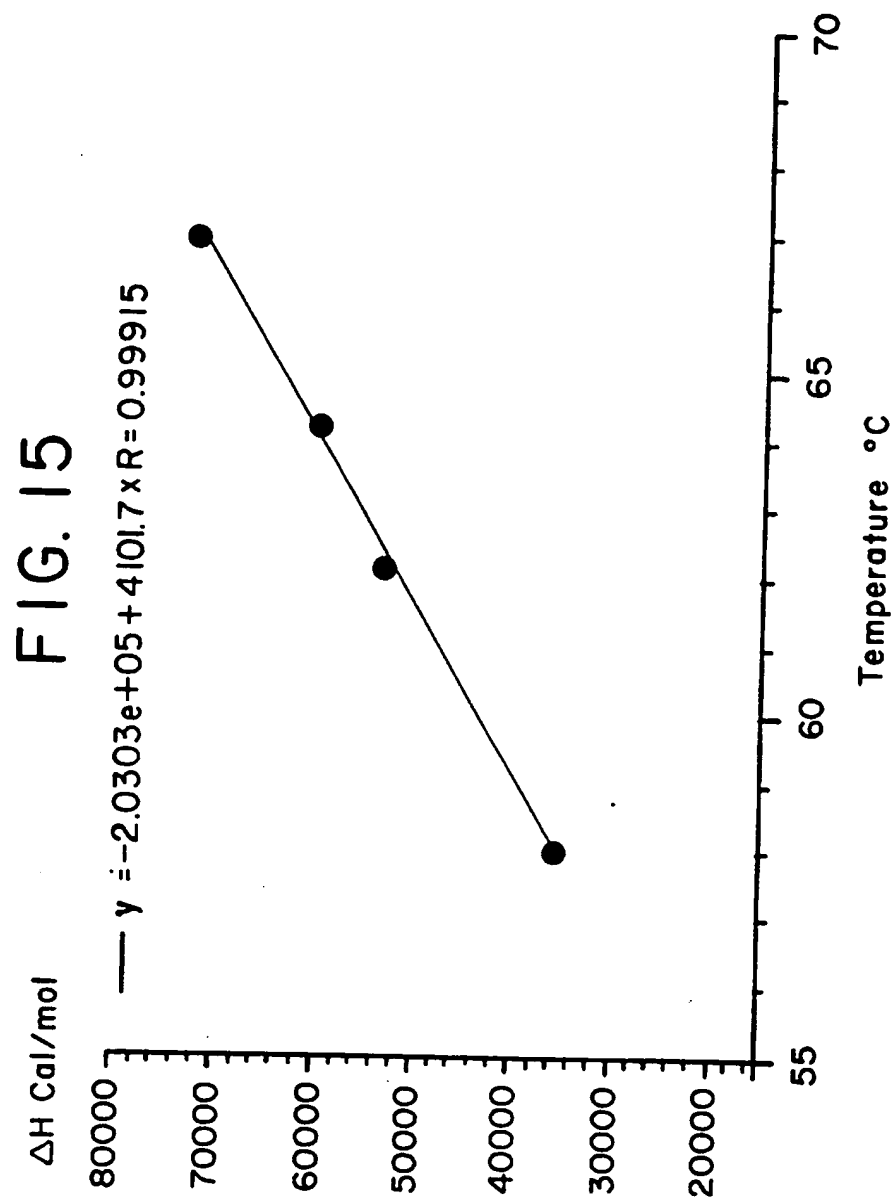


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FIG. 14B

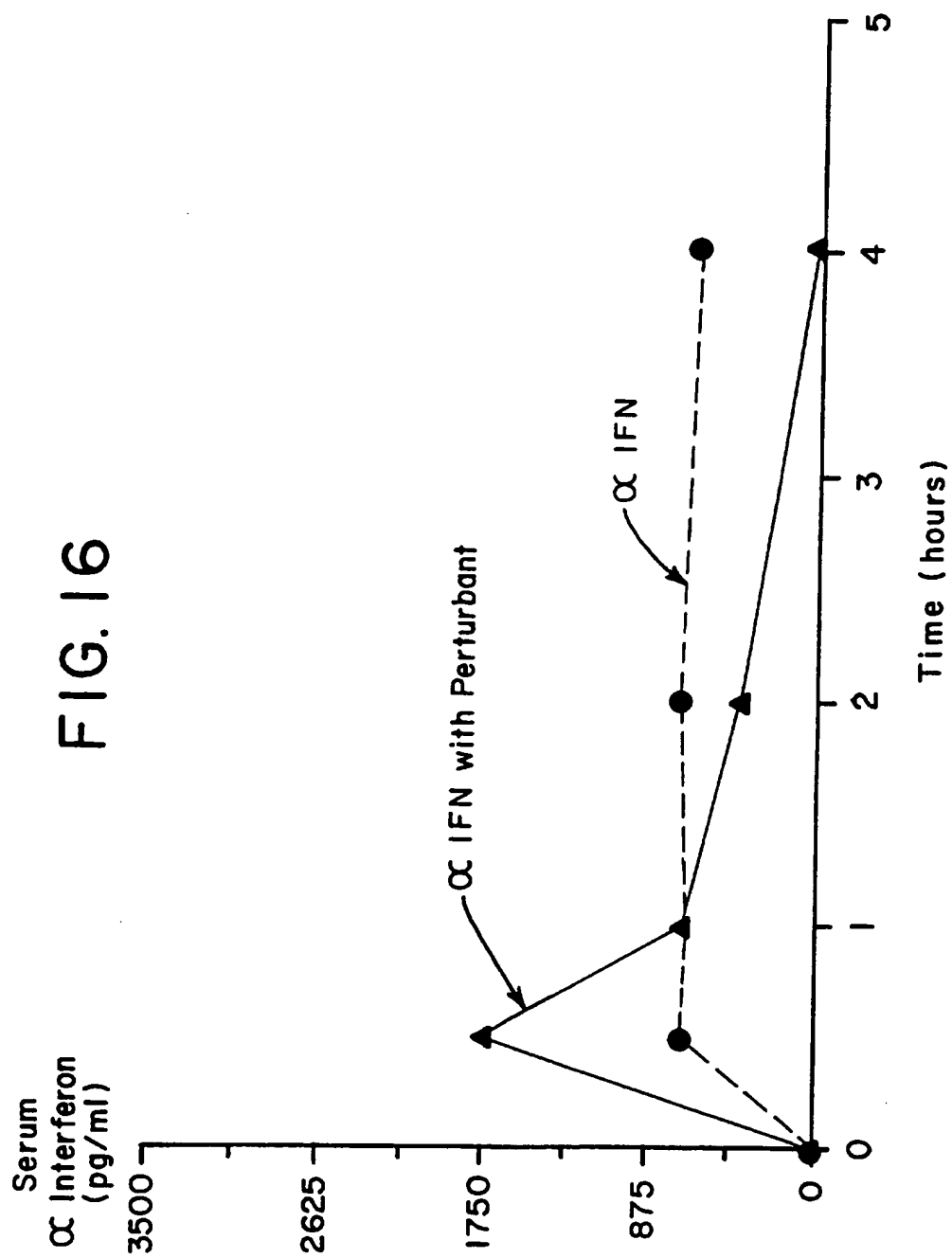


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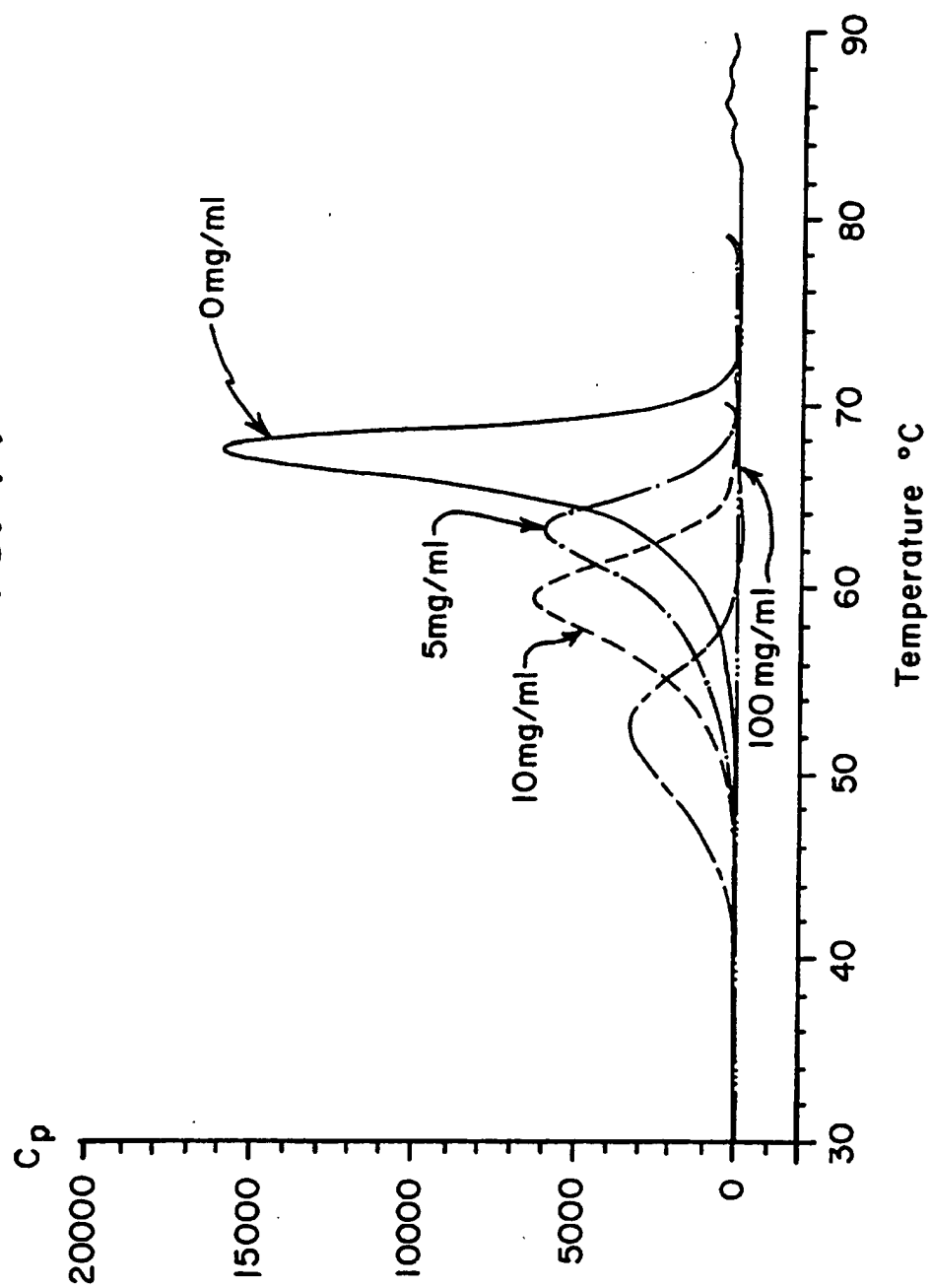


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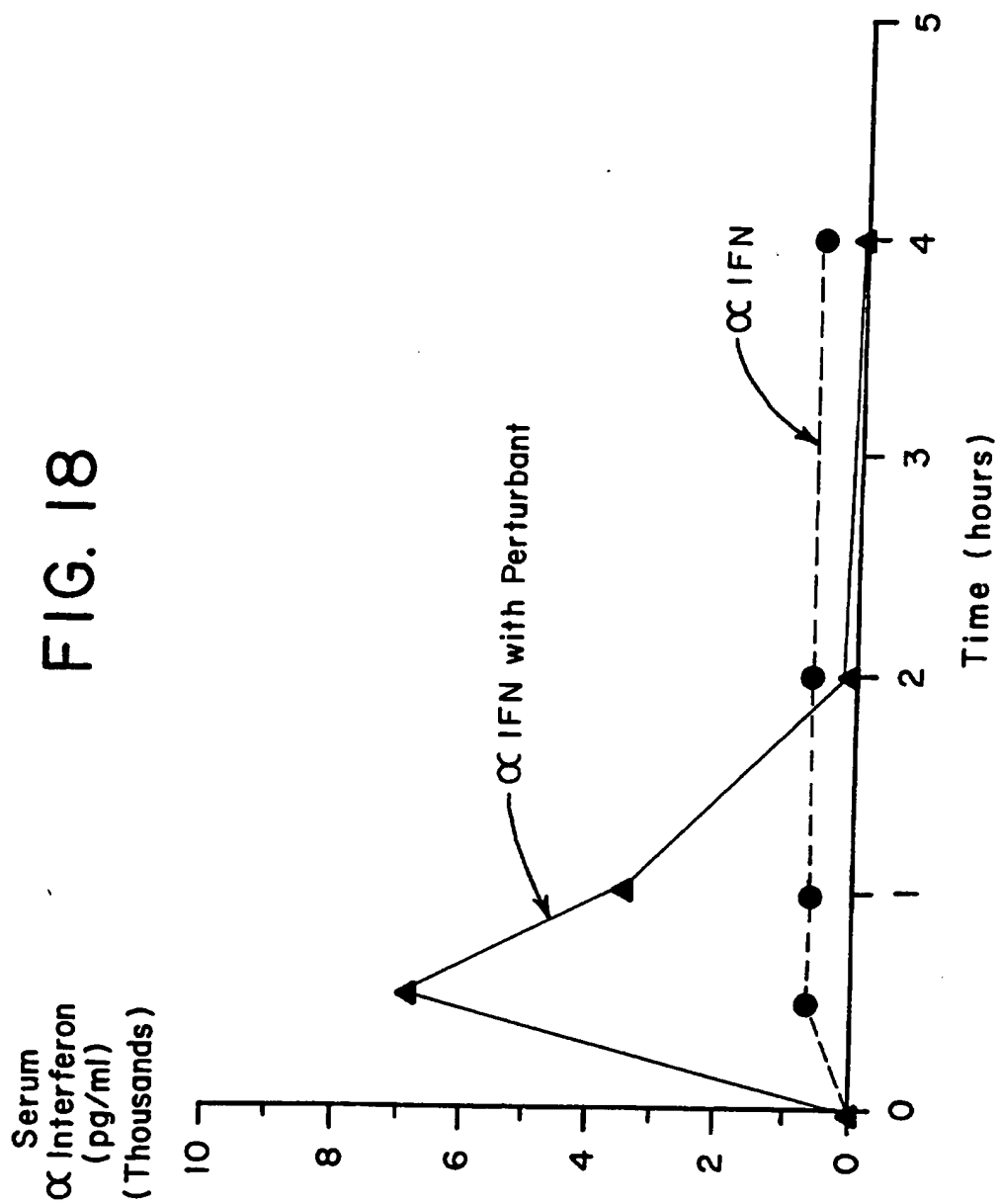


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FIG. 17

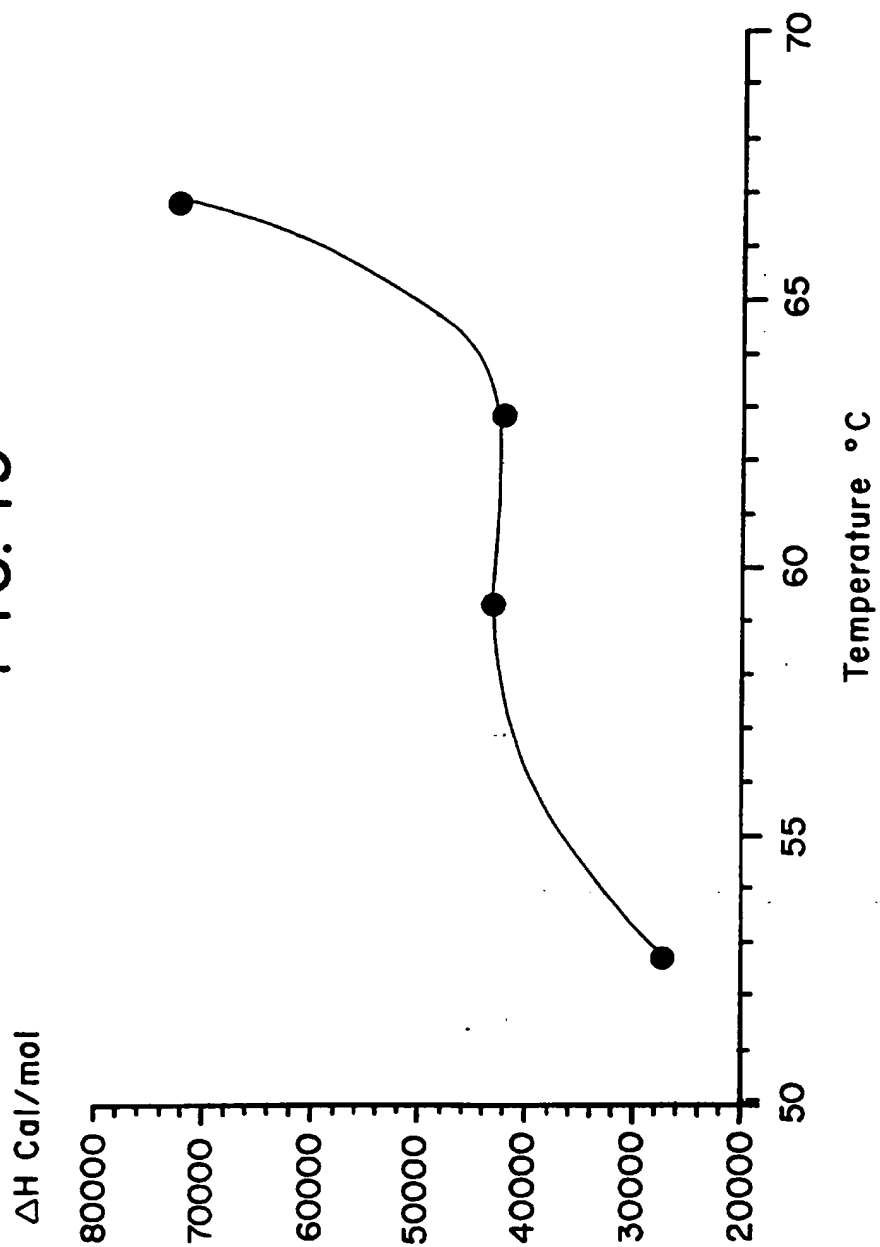


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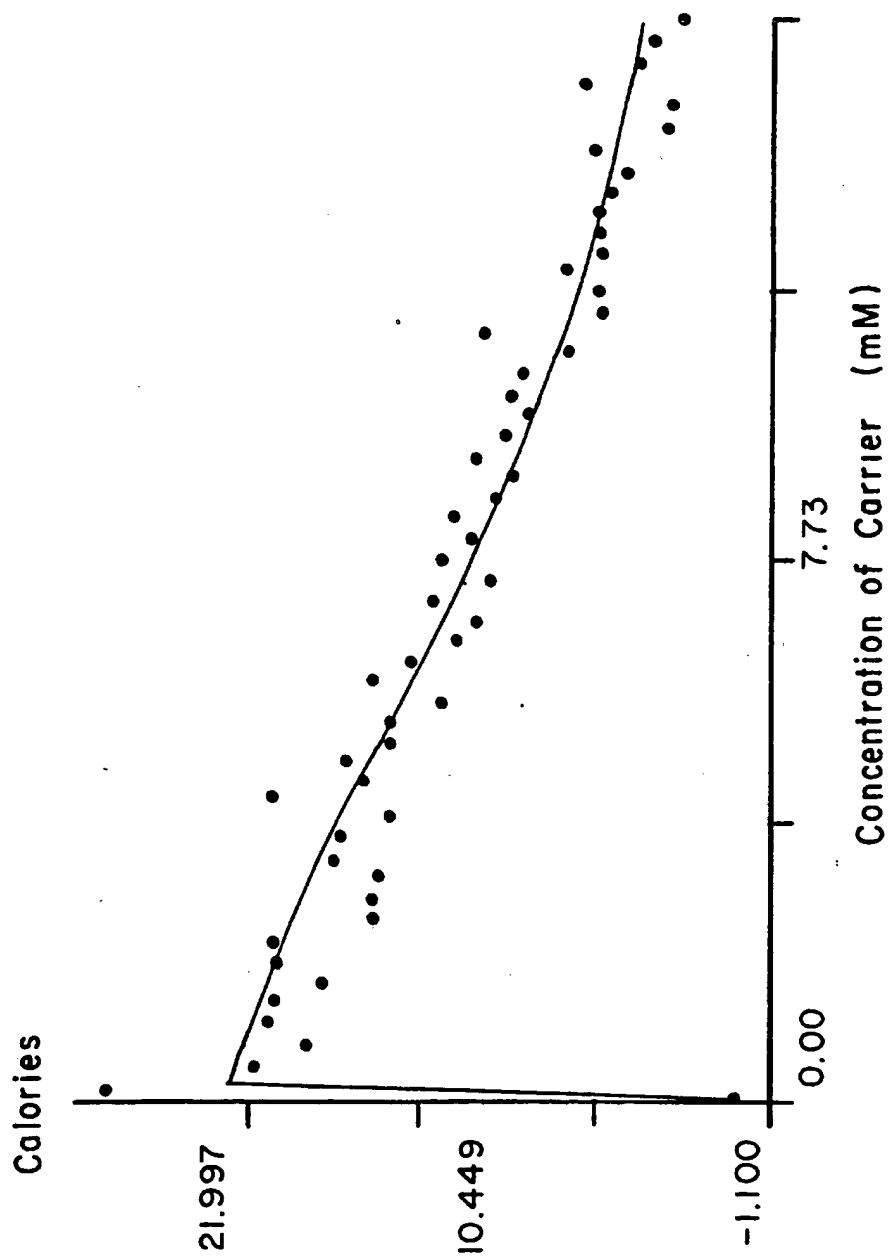
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FIG. 19



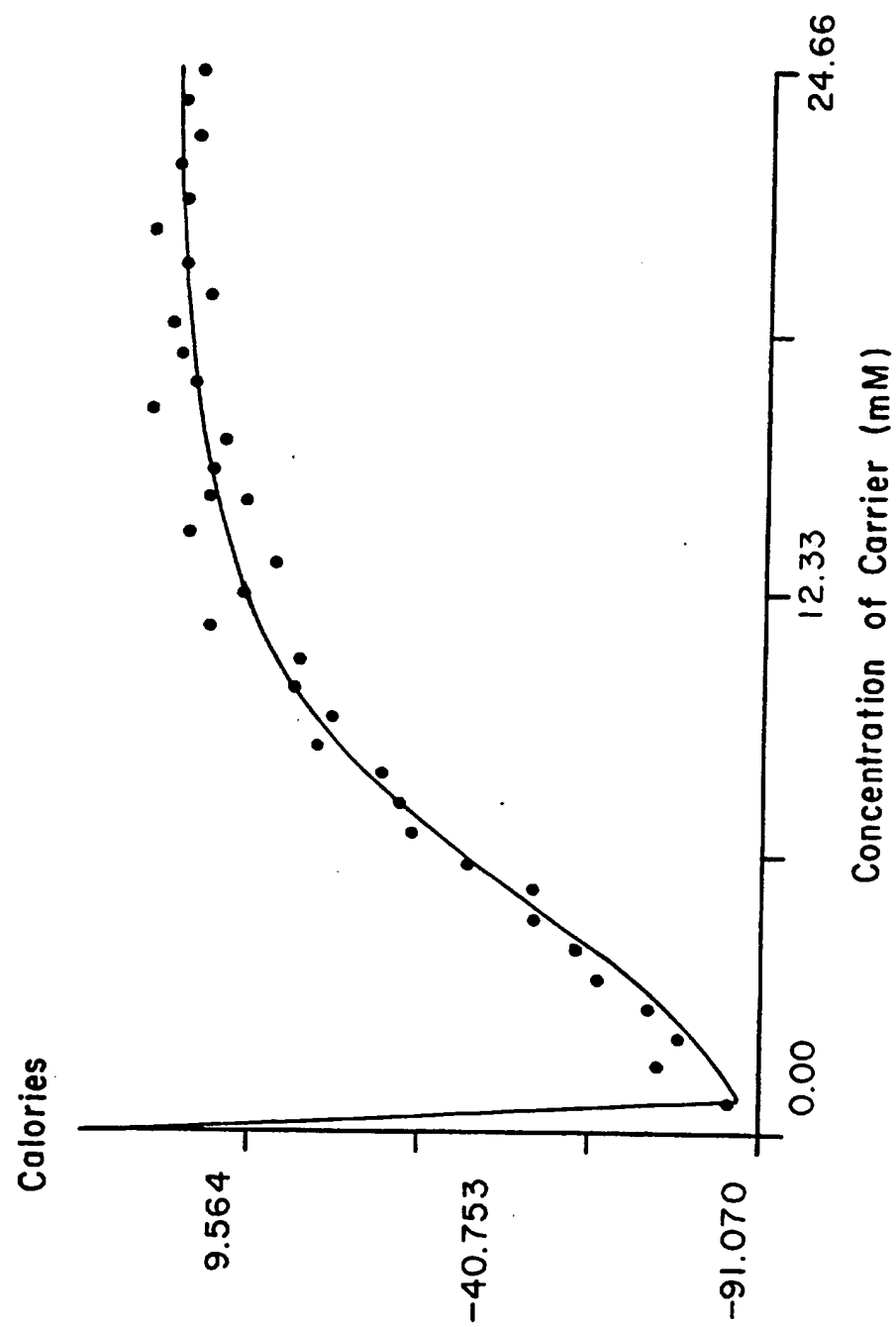
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FIG. 20



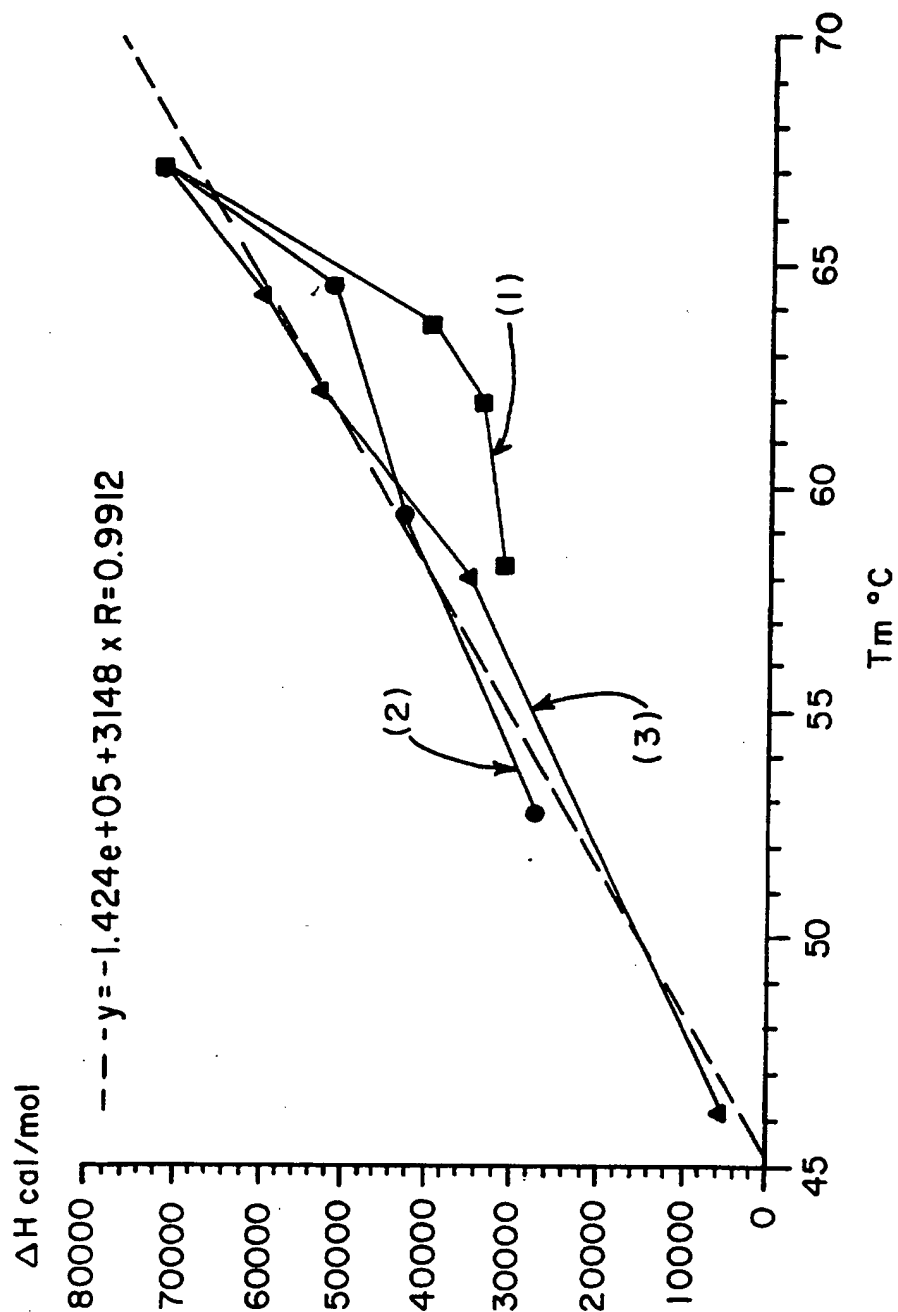
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FIG. 21



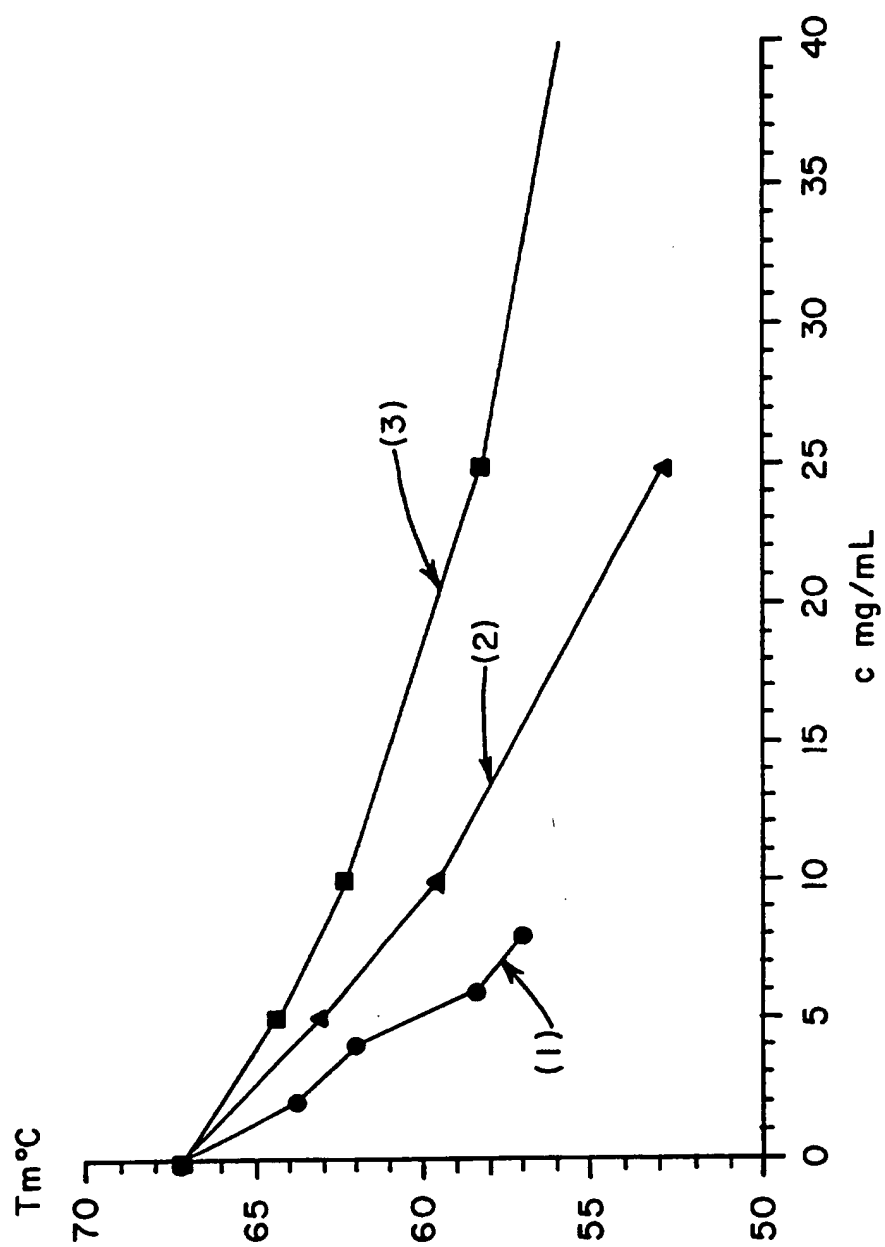
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FIG. 22



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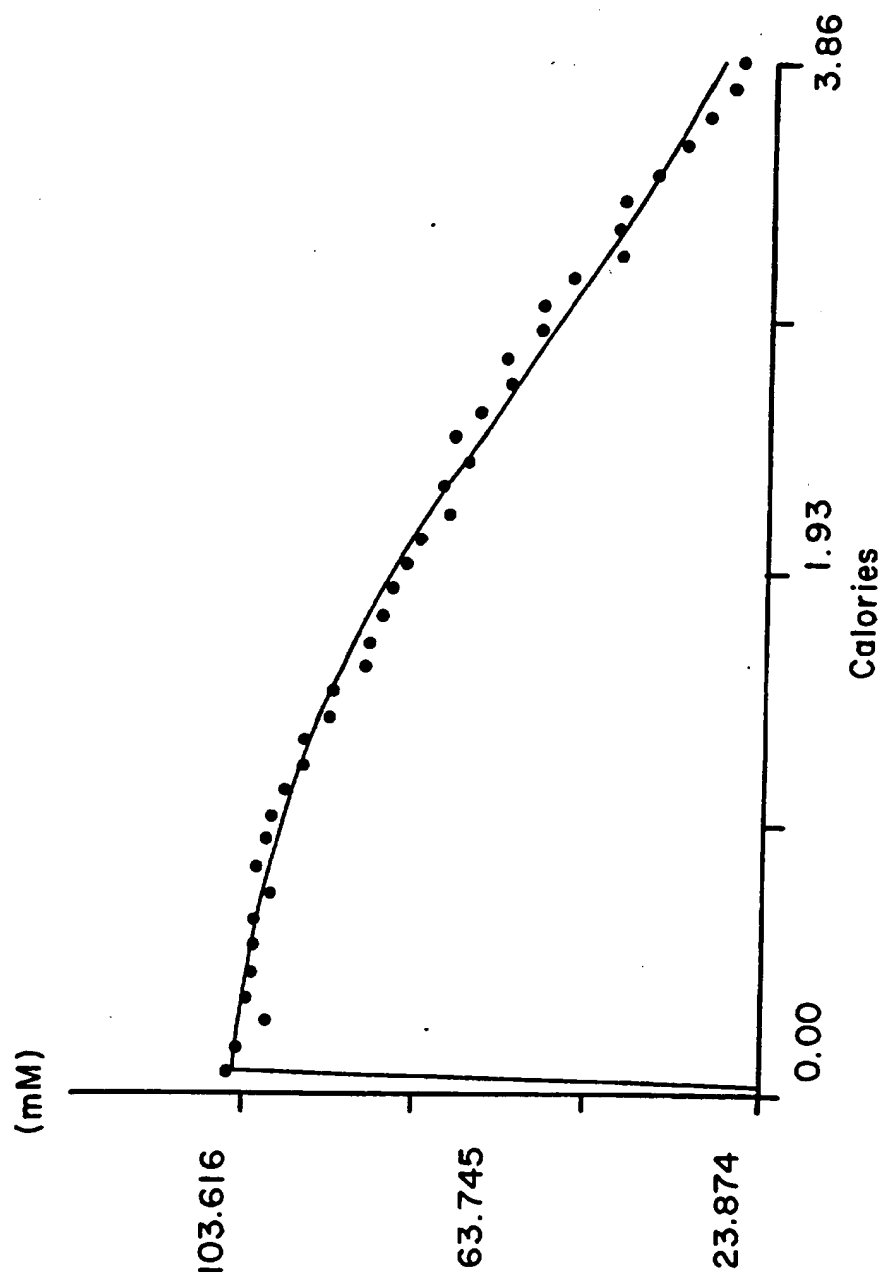
FIG. 23



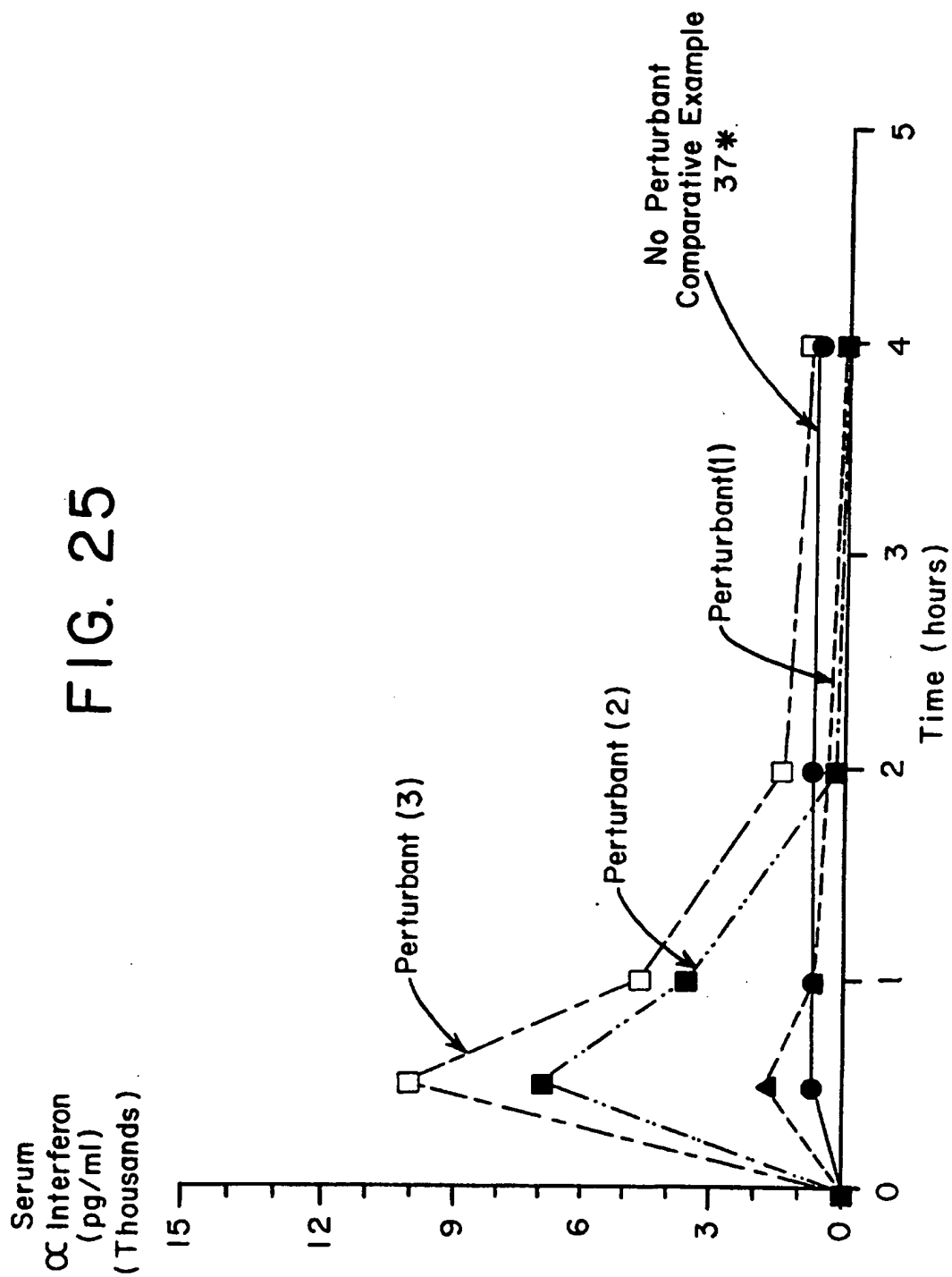


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FIG. 24

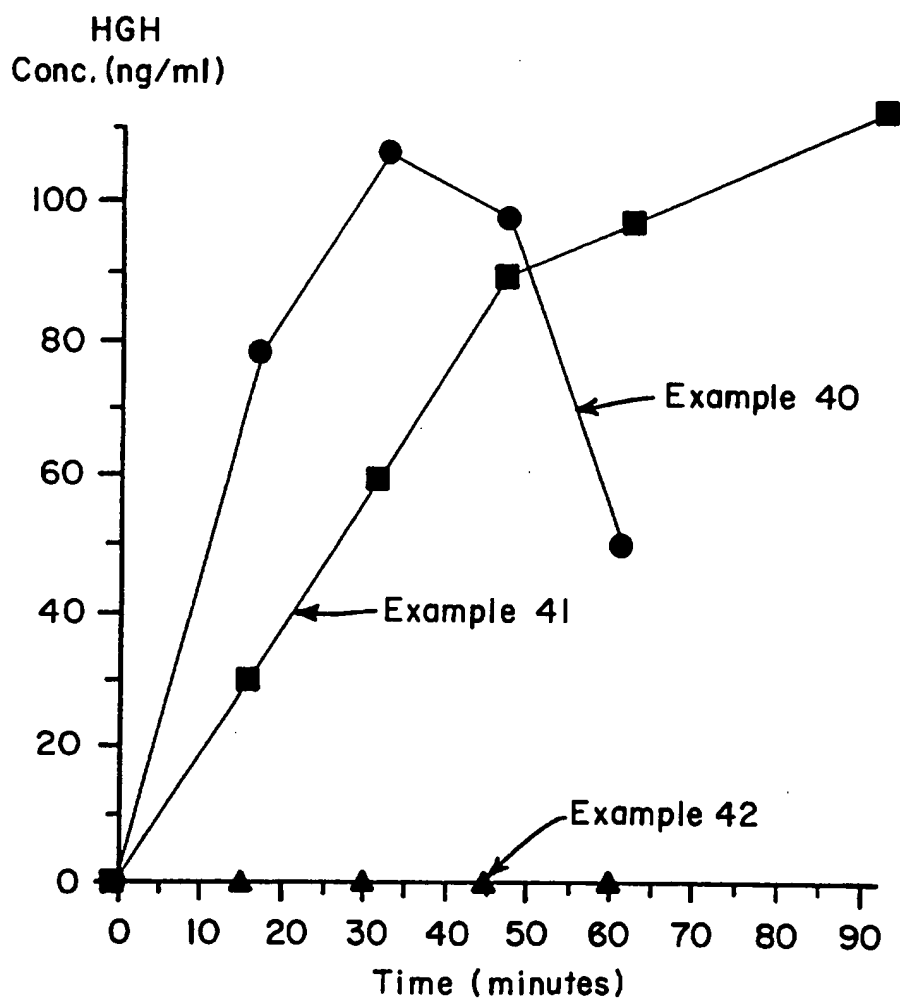


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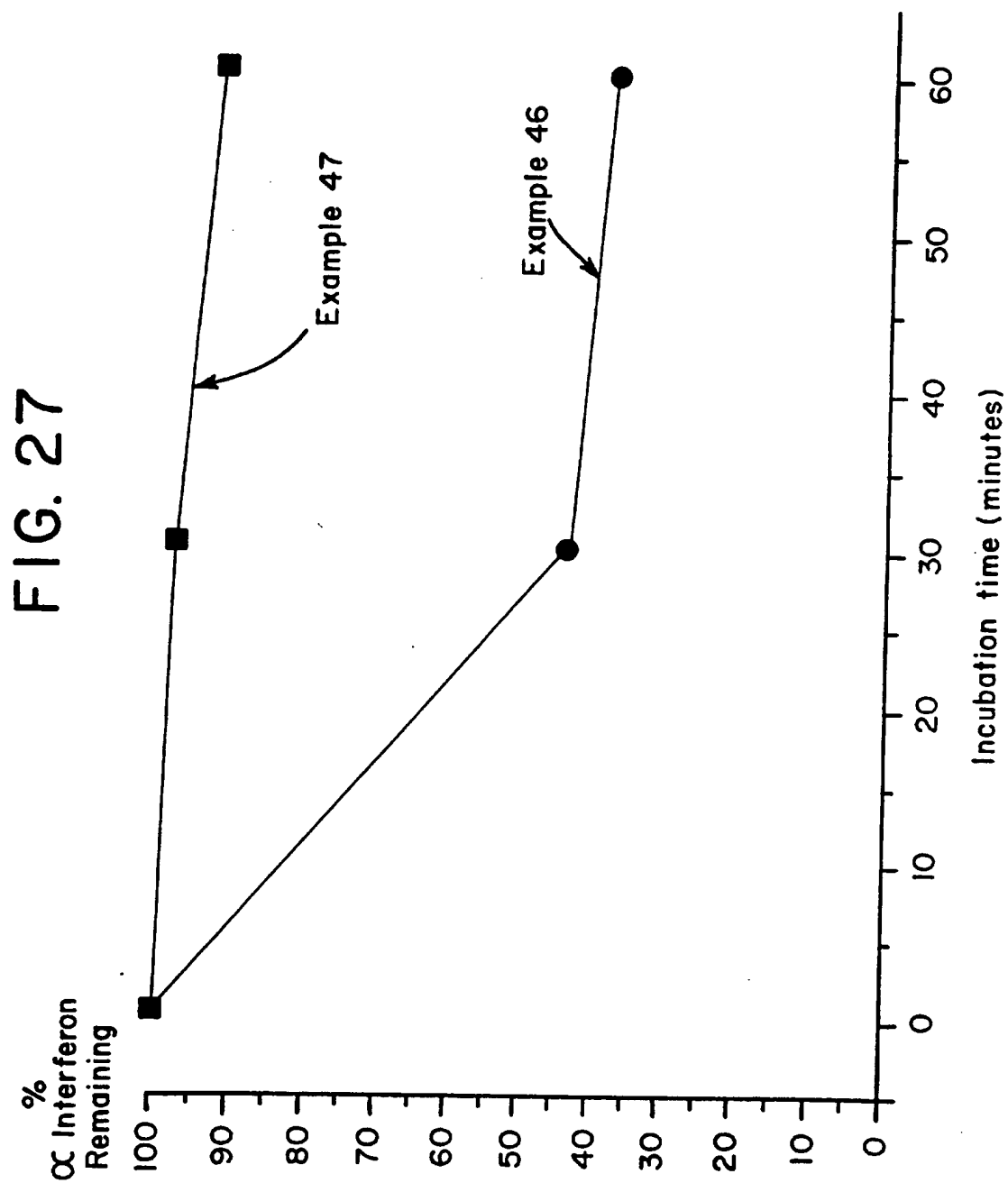


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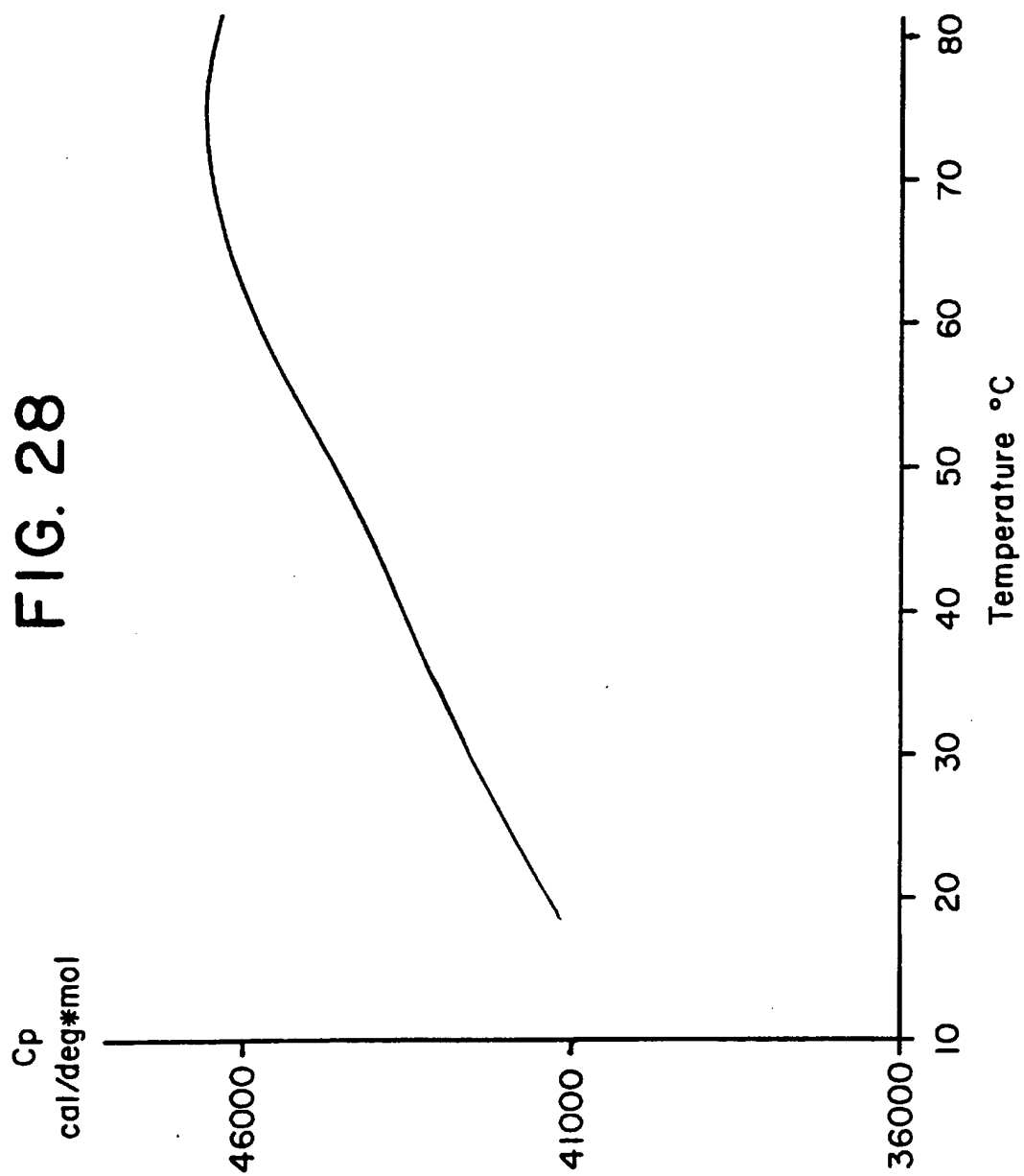
FIG. 26



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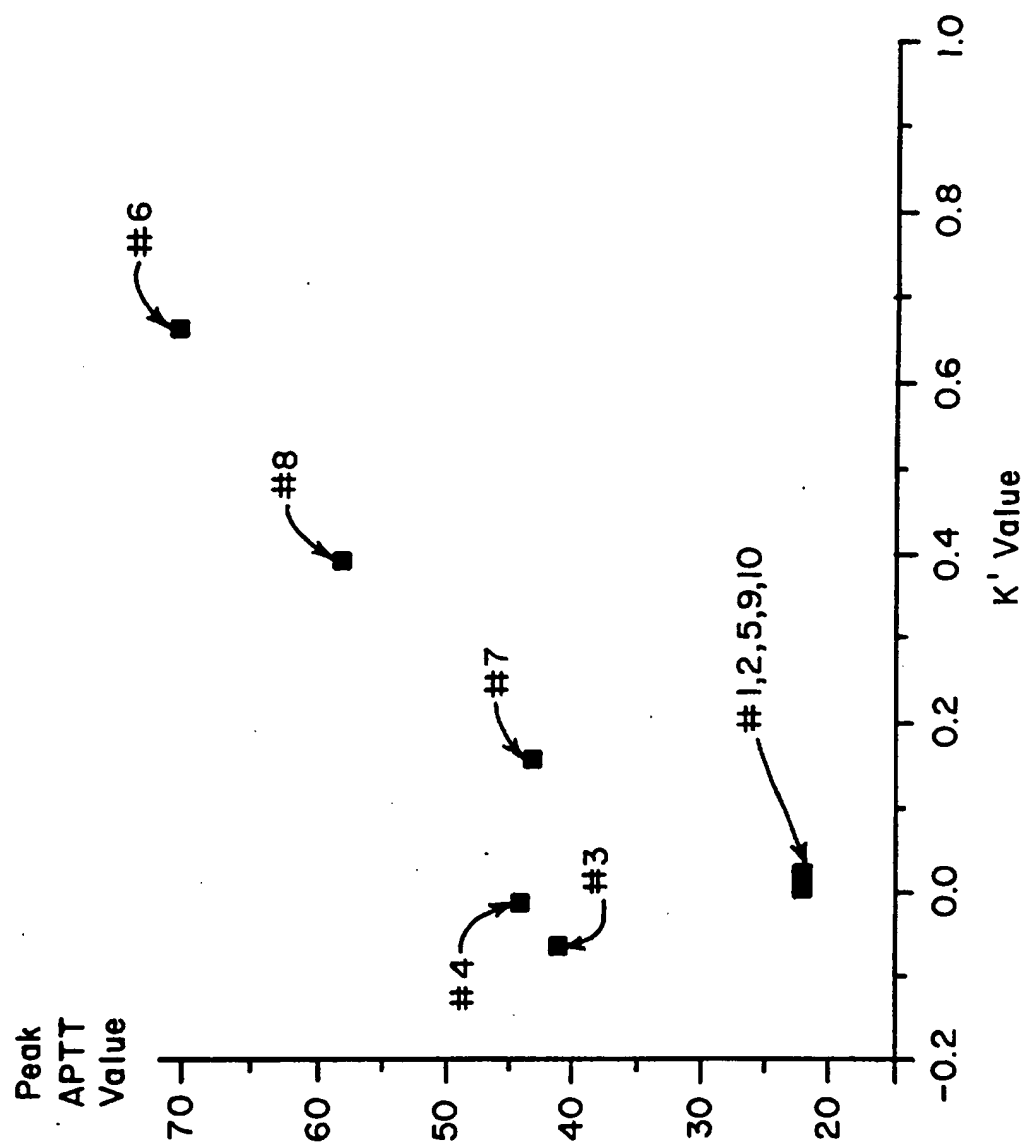


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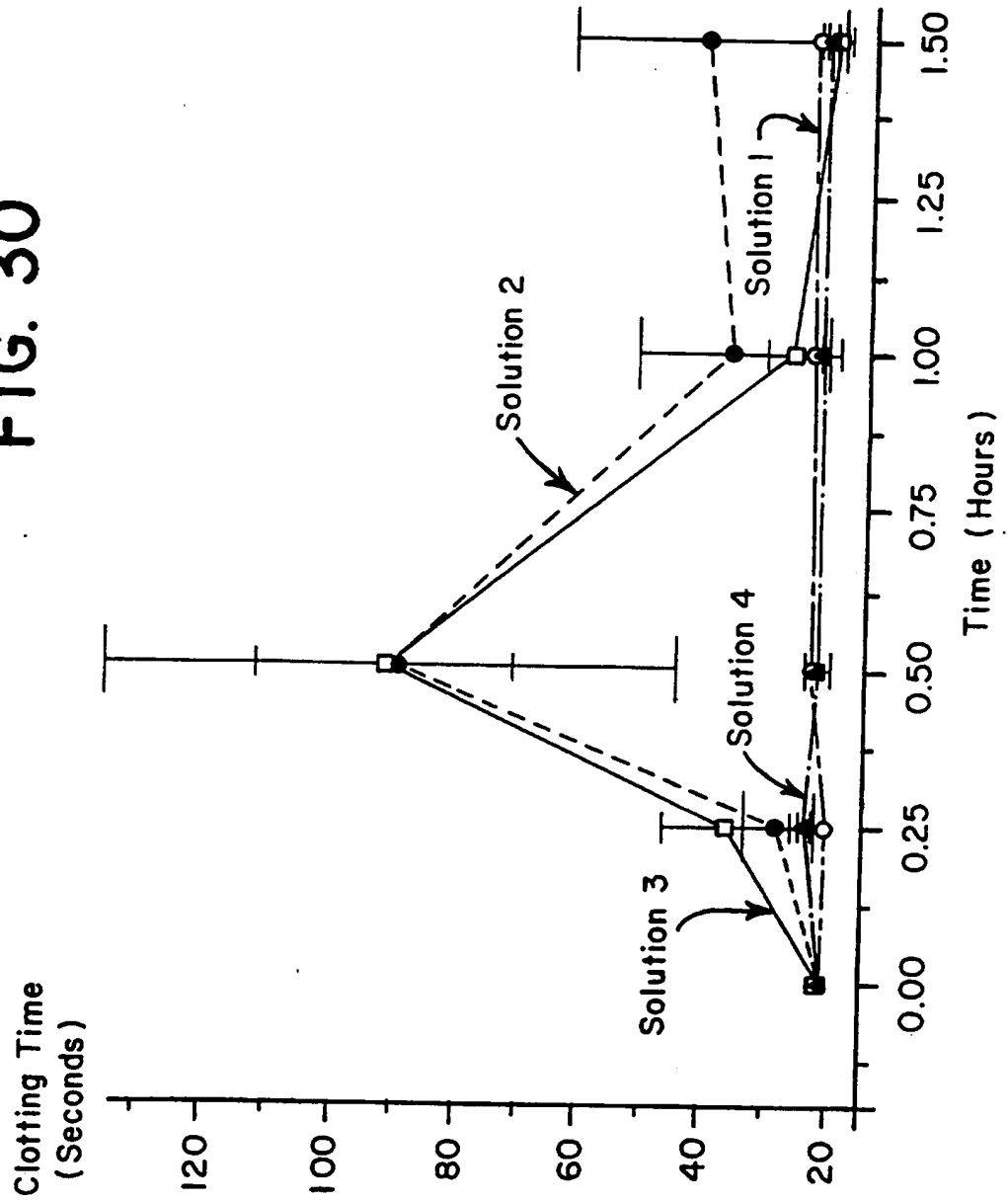
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FIG. 29



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FIG. 30



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/14598

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/16, 9/50

US CL :424/490

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/490, 488, 489, 491

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,455,335 (KAHNE) 03 October 1995, See entire document.	1-23
Y	US, A, 5,149,782 (CHANG) 22 September 1992, See entire document.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 FEBRUARY 1996

Date of mailing of the international search report

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